Volume 2

Plant Mineral Nutrition and Pesticide Management

Edited by
Ramdane Dris and S. Mohan Jain

Production Practices and Quality Assessment of Food Crops

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PREFACE

Plants require nutrients in order to grow, develop and complete their life cycle. Mineral fertilizers, and hence the fertilizer industry, constitute one of the most important keys to the world food supplies. There is growing concern about the safety and quality of food. Carbon, hydrogen and oxygen, which, together with nitrogen, form the structural matter in plants, are freely available from air and water. Nitrogen, phosphorus and potassium, on the other hand, may not be present in quantities or forms sufficient to support plant growth. In this case, the absence of these nutrients constitutes a limiting factor. The supply of nutrients to the plants should be balanced in order to maximise the efficiency of the individual nutrients so that these meet the needs of the particular crop and soil type. For example, it should be noted that EU-wide regulations are not designed to govern the specific details of mineral fertilizer use. Although plants receive a natural supply of nitrogen, phosphorus and potassium from organic matter and soil minerals, this is not usually sufficient to satisfy the demands of crop plants. The supply of nutrients must therefore be supplemented with fertilizers, both to meet the requirements of crops during periods of plant growth and to replenish soil reserves after the crop has been harvested.

Pesticides are important in modern farming and will remain indispensable for the foreseeable future. Without them it would be practically impossible to produce the enormous quantities of food that are required to feed the world's growing population. Multi-residue analysis of pesticides is applied routinely in food control laboratories around the world, especially in the control of fruits, vegetables, and cereals, since they are generally produced using direct applications of pesticides. Technical aspects of the application of pesticides and other agricultural inputs are in many countries of the world neglected and on field level unknown. Studies have shown convincingly that most farmers in developing countries can not handle highly hazardous pesticides in an acceptable manner. European Proficiency Tests 1996/97 (incurred pepper and spiked apple), Swedish NFA Inter-calibration Test 1995 (incurred grapes), and Spanish MAFF Inter-laboratory Tests 1994/95/96 (spiked and incurred peppers, and incurred lettuces). Pesticides must be applied with utmost care in the most efficient manner to protect crops and farm animals, while leaving the lowest possible residues in food and the environment. The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) has, since its inception in 1963, updated on a regular basis the scientific principles and methods by which it assesses pesticides. However, its operating procedures and resources have remained static despite the huge increase in work load associated with the evaluation of pesticides today compared to the time of its inception forty years ago.

Nine chapters are included in this book, which are: Environmental and Biological Monitoring of Exposure to Pesticides in occupationally Exposed Subjects; Crop Quality Under Adverse Conditions: Importance of determining the Nutritional Status; Phosphorus Management in French Bean (*Phaseolus vulgaris* L.); Nutrition and Calcium Fertilization of Apple Trees Diagnosis, Prediction and Control of Boron Deficiency in Olive Trees; Boron-Calcium Relationship in Biological Nitrogen

Fixation Under Physiological and Salt-Stressing Conditions; Lime-Induced Iron Chlorosis in Fruit Trees; Si in Horticultural Industry; Biological Monitoring of Exposure to pesticides in the General Population (Non-Occupationally Exposed to Pesticides).

In this book, we will cover various aspects on mineral nutrition, fertilizers and pesticide management to improve agricultural production, yield and to amelioration of soil fertility. The production of good quality food can not be achieved without the strict control of the quality and the use of pesticides. There is a need to increase research and development facilities to focus on new product development, seeking solutions to environmental problems and making more efficient use of applied nutrients and pesticides.

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ENVIRONMENTAL AND BIOLOGICAL MONITORING OF EXPOSURE TO PESTICIDES IN OCCUPATIONALLY EXPOSED SUBJECTS

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1. INTRODUCTION

Exposure to pesticides affects much of the population, including persons who are occupationally exposed as well as the general population, which may have contact with pesticides through domestic use, consumption of contaminated food and drink or by living in agricultural areas or areas treated for reasons of public health.

From the occupational viewpoint, exposure to pesticides regards the industrial, agricultural, public health (pest and rat control) and veterinary sectors (treatment of animals).

The major agricultural tasks carried out in the field or in greenhouses or tunnels, include mixing, loading, distribution, maintenance and repair of machinery and tools, and re-entry of treated areas. During loading and mixing, exposure depends on the type of formulation (solid, liquid), the size of solid particles, the size of the container, the number of operations carried out during the work shift, the quantity of formula and method of loading (use of soluble bags helps to reduce exposure levels). During distribution, exposure levels depend on the type of machines, the technique used, the size of aerosol particles and the quantity of pesticide distributed, which in turn depends on the size of the area to be treated and the time of application.

Re-entry tasks include all manual and mechanical operations carried out on plants previously treated with pesticides. They include harvest (fruit, vegetables and flowers), irrigation, thinning, staking, spacing, securing and so forth. In this case exposure depends on the quantity of pesticide applied and the interval elapsing since treatment. The term 're-entry period' (Goh et al., 1986) means the interval between distribution of pesticide and re-entry of the treated area necessary for safe manual operations without means of protection. Re-entry times have been established for various pesticides by monitoring decay of pesticide residues on leaves. Variables affecting pesticide break down include the physicochemical properties of the active ingredient, its capacity to be absorbed by plants, as well as microclimatic and environmental factors such as temperature and solar radiation (Brouwer et al., 1992a).

Activities and operations carried out in the chemical industry include synthesis and packaging of active ingredient, formulation, packaging of formula and maintenance and repair of machinery and tools. In such cases, workers are usually exposed to few active ingredients relatively constantly for long periods.

Agricultural activities in confined spaces and formulation of commercial products

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(mixing of active ingredients with excipients) are intermediate in character between farming in the open field and industrial activity. The work is done in a controlled microclimate and there is contact with many different products formulated in cycles.

Knowledge of exposure levels is a first step in the risk evaluation process and measurement may be done in different ways (predictive models, existing measurements, measurement under experimental conditions, representative sampling).

Predictive models of exposure are used when direct measurements cannot be made or are difficult or costly. They consist of mathematical representation of pesticide dispersal in the environment, based on its physicochemical properties and partial measurements. If not carefully validated, models may be much less accurate than direct evaluation of exposure. Although they are widely used in epidemiology to estimate environmental exposure, they are rarely used to evaluate occupational risk.

The use of existing measurements has the advantage of exploiting direct measurements reported in the literature and obtained during evaluation of environmental or occupational exposure. This method is therefore less costly than studies carried out for the specific purpose. An important application is to predict exposure to compounds that are not determined but which are used in the tasks monitored. A condition of this technique is that exposure be determined more from the physical properties of the formula, and from methods and conditions of use, than from the chemical nature of the pesticide.

Use of measures obtained under experimental conditions may lead to large errors because of the difficulty of reproducing in the laboratory real multiple conditions of field exposure (weather, climate and process techniques).

Representative sampling is the best strategy for evaluating exposure, its main problem being cost and sometimes practical considerations.

2. RESPIRATORY EXPOSURE

For certain types of active ingredient, method of distribution, work environment, climate (or microclimate) and occupational task, pesticides may be dispersed in the air as aerosols and/or vapour.

Direct methods of evaluating respiratory exposure proposed by Durham and Wolfe in 1962 (Durham and Wolfe, 1962) employ a respirator interfaced with a pad which may be of various materials. Surgical gauze and alpha-cellulose have been used to sample substances in dry and liquid form, respectively. The pads intercept the total quantity of aerosol that would otherwise be inhaled by the worker. This quantity can be expressed as potential hourly dose by dividing by the time of exposure. The advantages of this technique are simplicity of use and the fact that the amount of aerosol trapped by the system depends on the real respiratory regime of the subject. Disadvantages are dampening of the pad by expired air which modifies capture efficiency and may lead to hydrolysis of the active ingredient. These limits and the difficulty of convincing workers to wear the respirator have meant that direct methods have largely been replaced by air sampling procedures.

2.1. Methods of air sampling

The strategy may involve personal and/or area air sampling, depending on whether the sampler is worn by the operator (near the mouth and nose) or whether it is installed in the work place. Personal air sampling is more suitable than area air sampling for evaluation of exposure of workers. Temperature and pressure of sampling must be recorded in order to correct and standardise the volumes of air sampled.

The duration of sampling is determined by changes in concentration that may occur in time, by sampling flow and by limits of detection (LOD) of analytical methods. Short sampling periods repeated during the work shift may provide a good approximation of real exposure conditions. For example, a worker may be engaged in various tasks during the work shift and each of these can be monitored. Mixing and loading are tasks which may only last a few minutes, but it may be useful to sample them separately as there may be large variations in exposure. If the sampling substrate is changed with each change of task (Brouwer et al., 1993)), daily exposure is represented by the time-weighted mean of pesticide concentrations detected in each period. In other cases (Aprea et al., 1994a; Aprea et al., 1995; Aprea et al., 1998; Aprea et al., 1999b; Aprea et al., 2001a; Aprea et al., 2002; Fenske and Elkner, 1990), sampling is made to cover the whole work shift or a shorter but representative interval of the working day.

During synthesis and packaging of active ingredient, work proceeds in a continuous manner with the same products. In this case evaluation of exposure should be directed at all active ingredients and excipients dealt with in each production cycle in a differentiated way and should be repeated at different times of year for the various substances.

Interpretation of exposure data obtained in the field (external air) is more difficult than interpretation of values measured in a confined environment because in the former case, the results are affected by variables such as wind (direction and speed), temperature and thermal inversions.

2.2. Sampling of pesticides dispersed as aerosol

Of the various techniques (van Dyk and Visweswariah, 1975) of sampling aerosols (filtration, bubbling, impact and granulometric separation, sedimentation, electrostatic precipitation, thermal precipitation, centrifuge methods), the most widely used is filtration with cellulose ester or nitrate membranes or fiberglass filters. Table 1 shows recent papers on capture of particulate by air filtration. The sampling substrates reported in the official methods of the U.S. National Institute for Occupational Safety and Health (NIOSH), the Occupational Safety and Health Administration (OSHA) and the Environmental Protection Agency (EPA) are also indicated.

2.3. Sampling of pesticides dispersed as vapour

The most widely used methods are adsorption on solid materials in vials and absorption in liquids by bubbling.

Table 1. Systems of air filtration sampling used in official and other methods.

Pesticide	Sampling substrate	Flow (l/min)	References
2,4-D	Glass fiber filter	4	Abbott et al., 1987
2,4-D	Glass fiber filter binding free	1–3	NIOSH, 1994a
2,4-D, MCPA	Glass fiber filter	2	Aprea et al., 1995
Acephate	Mixed cellulose ester filter	4	Maroni et al., 1990
Acephate, Benomyl, Carbaryl, Diazinone Chlorothalonil, Dicofol	Glass fiber filter	2	Leonard and Yeary, 1990
Alachlor	Solid phase extraction filter	1	NIOSH, 1998a
Captan	Glass fiber filter	2[d]	de Cock et al., 1995
Carbaryl	Glass fiber filter binding free	1–3	NIOSH, 1994c
Chlorpyrifos	Glass fiber filter	2[e]	Fenske and Elkner, 1990
Delthametrrin, Fenvalerate	Mixed cellulose ester filter	2[f]	Zhang et al., 1991
Ethylenethiourea	Glass fiber filter	2	OSHA, 1992
Ethylenethiourea	Polivinylchloride filter	1–3	NIOSH, 1994e
Ethylenethiourea	Mixed cellulose ester filter	1–3	NIOSH, 1994e
Ethylenethiourea	Mixed cellulose ester filter	2–3	Kurttio and Savolainen, 1990; Kurttio et al., 1990
Ethylenethiourea, Mancozeb, Dimethoate	Glass fiber filter	2.8[a]	Aprea et al., 1998
Fenvalerate, Delthametrrin	Glass fiber filter	2–10	He et al., 1988
Fenvalerate, Delthametrrin	Mixed cellulose ester filter	2–10	He et al., 1988
Fosetil-Al	Glass fiber filter	2	Fenske et al., 1987
Maneb	Mixed cellulose ester filter	2	OSHA, 1996
Metomyl, Pirazophos, Fenarimol, Captan, Endosulfan, Carbendazim	Glass fiber filter	14[c]	Stevens and Davies, 1981
Metomyl, Captan, Endosulfan, Carbendazim, Pirazophos, Fenarimol	Mixed cellulose ester filter	3	Stephanou and Zourari, 1989
Picloram	Glass fiber filter	1	OSHA, 1990c
Piretro	Glass fiber filter	1–4	NIOSH, 1994d
Propetamphos	Glass fiber filter	1	OSHA, 1989c
Propoxur	Glass fiber filter	2[b,c]	Brouwer et al., 1993
Temephos	Glass fiber filter	1	OSHA, 1990e
Thiophanate-methyl	Glass fiber filter	1	OSHA, 1989e
Thiram	Politetrafluoroethylene filter	1–4	NIOSH, 1994b
Thiram, Zineb, Thiophanate-methyl	Mixed cellulose ester filter	2[b]	Brouwer et al., 1992b
Zineb	Mixed cellulose ester filter	2	OSHA, 1996

[a] Inhalable fraction with 7 mm reduction cone.
[b] Inhalable fraction with IOM sampler.
[c] Two filters in series.
[d] Inhalable fraction with 6 mm reduction cone.
[e] Inhalable and respirable fractions (cyclone).
[f] Granulometry was determined by impact at a flow of 20 l/min.

The main features of an adsorbent are low flow resistance, high adsorption capacity, inertness, resistance to fracture and easy release of the adsorbed substance for analysis (van Dyk and Visweswariah, 1975).

Liquids used to absorb pesticides must not foam or be inflammable, volatile or viscous. They should ensure ready solubilisation of the pesticide in the vapour phase and should be chemically stable and non corrosive (van Dyk and Visweswariah, 1975). Ethylene glycol has been used for lindane, dieldrin and DDT, and n-butanol, toluene, hexane and water for diclorvos (van Dyk and Visweswariah, 1975). Ethylene glycol has proved to be an excellent absorbent for most pesticides but its use is limited by the fact that it absorbs atmospheric humidity which may lead to hydrolysis of active principles.

Liquids that react with the substance to be sampled have also been used, for example monoethanolamine reacts with diclorvos to form a coloured compound that can be analysed by spectrophotometry (van Dyk and Visweswariah, 1975). Reactive liquids (2-methoxymethanol/NaOH, 1:1 v/v) have also been used for parathion and methylparathion (van Dyk and Visweswariah, 1975). Solutions of cholinesterase have been used as absorption liquid for parathion and demeton (van Dyk and Visweswariah, 1975).

The most recent systems for sampling pesticide vapours are shown in Table 2.

2.4. Combined sampling systems

A combined or two-stage system consists of two or more sampling units linked in series in order to sample various physical forms of airborne pesticide simultaneously present in a work environment or that may form by stripping in the first system as an effect of the air flow. Systems containing more than one unit of the same

Table 2. Sampling systems based on adsorption/absorption used in official and other methods.

Pesticide	Sampling substrate	Flow (l/min)	References
2,4-D, Diclorprop, Picloram	Florisil	0.2	Libich et al., 1984
2,4-D, MCPA, Diclorprop, Mecoprop	Water	1	Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b
Chlorpyrifos, Carbaryl, Permethrin	PUF**	2	Byers et al., 1992
Cipermethrin	ORBO 42	2	Wright et al., 1993
Deltamethrin, Dicofol	Hydrated Florisil		Mestres et al., 1985
Dimethoate	Amberlite	1	Aprea et al., 1998
Fluvalinate, Dicofol, Chlorpyrifos, Etazol	PUF**	3	Stamper et al., 1989
Chlorinated and phosphoric ester insecticides	PUF**	1–5	EPA, 1987b
Mevinfos	XAD-4	0.2–1.5	Kangas et al., 1993
Pentachlorophenol	XAD-7*	0.2	OSHA, 1982

* The device consists of two vials containing XAD-7 disposed in series.
** Polyurethane foam, the substrate also samples particulate but its efficiency is not known.

type (two vials in series or two membranes in series) have also been used to collect all the compound, when low capture efficiency makes it impossible to collect it all with a single unit (Brouwer et al., 1993; OSHA, 1982; Stephanou and Zourari, 1989). Recently used combined systems are shown in Table 3.

When combined systems are used, exposure is obtained summing the concentrations detected in the various serial units.

2.5. Comparison with environmental limits

The American Conference of Governmental Industrial Hygienists (ACGIH, 2002) has published threshold limit values (TLVs) for various pesticides. Similarly OSHA, NIOSH and other government and non government bodies of different countries (Australia, Belgium, Denmark, France, Germany, Switzerland, UK, Finland, Japan etc.) have published various types of limits for respiratory exposure to pesticides, sometimes with the notation 'skin' to indicate the possibility of transcutaneous exposure.

In the case of industrial occupations, respiratory exposure can be compared with limit values. Farm work, on the other hand, has characteristics that make comparison with limit values, if they exist, almost impossible:

– pesticide use is concentrated in short periods repeated during the year (intermittent exposure);
– more than one substance having different toxicological properties may be used simultaneously;
– tasks vary and are sometimes associated with cutaneous rather than respiratory exposure, or vice versa;
– pesticide use is characterised by qualitative and quantitative variations that may depend on agricultural factors, weather, and so forth.

2.6. Respiratory dose

To estimate respiratory dose using air sampling, the concentration of pesticide detected in personal air samples (RE = respiratory exposure) expressed in units of mass per cubic metre, is corrected for the volume of air inhaled by the subject during the period of exposure (T). This volume depends on pulmonary ventilation (PV) expressed in l/min which is in turn determined by the physical exertion required by the task undertaken. Table 4 shows lung ventilation values used by various authors to calculated respiratory dose (RD) for various occupational tasks.

The general formula used to calculate respiratory dose is:

$$DR(\text{mass}) = \frac{ER\ (\text{mass/m}^3) \cdot PV(1/\text{min}) \cdot T(\text{min})}{1000}$$

To calculate absorbed dose, the numerator of the formula is multiplied by PR%.

If personal protection such as a mask is not worn and if no specific studies exist, various authors use a PR of 100% (Aprea et al., 1998; Fenske and Elkner,

Table 3. Two-stage sampling systems of official and other methods.

Pesticide	Sampling substrate	Flow (l/min)	References
Alachlor	OVS*	0.2–1	NIOSH, 1998b
Aldicarb	OVS-2	1	OSHA, 1988b
Atrazine	OVS*	0.2–1	NIOSH, 1998b
Bendiocarb	OVS-2	1	OSHA, 1989a
Carbaryl	OVS-2	1	OSHA, 1987a
Chlordane	OVS-2	1	OSHA, 1987b
Chlorpyrifos	OVS-2	1	OSHA, 1986
Clorothalonil	M FV[c]/XAD4	1	Spencer et al., 1991
Clorothalonil	M EMC[a]/XAD2	2[b]	Brouwer et al., 1992b
Cyanazine	OVS*	0.2–1	NIOSH, 1998b
DDVP	OVS-2	1	OSHA, 1986
Demeton	EMC/XAD-2	0.2–1	NIOSH, 1994f
Diazinon	OVS-2	1	OSHA, 1986
Bendiocarb, Chlorpyrifos Diazinon	M FV[c]/chromosorb	1.7	Currie et al., 1990
Endosulfan	OVS-2	1	OSHA, 1988c
Fonophos	OVS-2	1	OSHA, 1989b
Phosphoric ester insecticides	OVS-2*	0.2–1	NIOSH, 1994g
Malathion	OVS-2	1	OSHA, 1986
Metolachlor	OVS*	0.2–1	NIOSH, 1998b
Metribuzin	OVS-2	1	OSHA, 1990a
Monocrotophos	OVS-2	1	OSHA, 1990b
Parathion	OVS-2	1	OSHA, 1986
Permethrin	M EMC[a]/tenax	0.5	Llewellyn et al., 1996
Dimethoate, Permethrin Pirimiphos-Methyl	M. EMC[a]/etanolo	0.3–0.5	Adamis et al., 1985
Propoxur	OVS-2	1	OSHA, 1987c
Pyrethrum	OVS-2	1	OSHA, 1988a
Resmethrin	OVS-2	1	OSHA, 1989d
Ronnel	OVS-2	1	OSHA, 1994
Simazine	OVS*	0.2–1	NIOSH, 1988b
Sulprofos	OVS-2	1	OSHA, 1990d
Azinphos-methyl, Chlorpyrifos, Diazinon, Dicrotophos, Disulfoton, Ethion, Ethoprop, Fenamiphos, Fonophos, Malation, Methamidophos, Parathion-methyl, Mevinphos, Monocrotophos, Parathion, Phorate, Ronnel, Sulprofos, Terbufos	OVS-2	1	Kennedy et al. 1994
Aldicarb, Benomyl, Captan, Carbaryl, Carbofuran, Chlorpropham, Diuron, Formetanate, Methiocarb, Metomyl, Oxamyl, Propham, Propoxur, Thiobencarb	OVS*	1	Kennedy et al. 1997
2,4-D	OVS*	0.2–1	NIOSH, 1998b
2,4-D 2-ethylhexyl estere	OVS*	0.2–1	NIOSH, 1998b
2,4-D 2-butoxyethyl estere	OVS*	0.2–1	NIOSH, 1998b
2,4-D Carbofuran, Trifluralin,	M PVC[d]/tenax	0.5	Guidotti et al., 1994

[a] Mixed cellulose ester membrane; [b] inhalable fraction (IOM sampler); [c] fiberglass membrane; [d] PVC membrane.
EMC/XAD-2 is a device consisting of a mixed cellulose ester membrane and vials containing XAD-2; OVS-2 is a commercially available device consisting of a glass vial containing XAD-2 divided into two sections, 270 mg (front) and 140 mg (back), separated by polyurethane foam. The front section is held in place by a fiberglass filter fixed with a polytetrafluoroethylene ring.
* A device similar to that reported in OSHA method 62 except that the filter is not fiberglass but quartz fiber.

Table 4. Pulmonary ventilation (PV) values and pulmonary retention (PR) used in various studies to calculate respiratory dose.

Task	PV (l/min)	PR (%)	References
Spraying in greenhouse	14.2	100	Stephanou and Zourari, 1989
Pest control of buildings	29[a]	100	Fenske and Elkner, 1990
Formulation, bottling	Males 28.6[b]	100	Aprea et al., 1998
and/or packaging	Females 16.3[b]	100	
Harvesting flowers in greenhouse	20.8	40[c]	Brouwer et al., 1993
Mixing, loading and distribution	20		Fenske et al., 1987
Distribution	25		Kurttio and Savolainen, 1990; Kurttio et al., 1990
Harvest of tomatoes in greenhouse	16.7		Adamis et al., 1985
Mixing and loading	29[a]		Byers et al., 1992
Formulation	20.8		Maroni et al., 1990
Mechanical harvest of tomatoes	Females 16[d]	100	Spencer et al., 1991

[a] Reported by Durham and Wolfe (Durham and Wolfe, 1962) for light work; [b] reported by Taylor (Taylor, 1941) for light work; [c] value based on studies with volunteers (Machemer et al., 1982); [d] reported by EPA for light work (EPA, 1987a).

1990; Spencer et al., 1991; Stephanou and Zourari, 1989). Other authors (Brouwer et al., 1993) use PR values obtained in studies on volunteers (Machemer et al., 1982).

If respiratory protection is worn, personal sampling provides a measure of potential exposure. To estimate real exposure it is necessary to check whether respiratory protection is worn throughout the work shift and determine the protection it affords.

3. SKIN EXPOSURE

Skin contamination may occur as a result of immersion, deposition or surface contact. For example, immersion occurs when part of the skin is immersed in a container containing a mixture of pesticides to be dispersed on crops. In such cases, exposure depends on chemical concentration of pesticide, area of skin immersed and duration of exposure. It can be reduced if protective clothing is worn and is generally evaluated by biological monitoring or by means of models, rather than by direct measurement. A special situation arises when a worker wears garments, such as gloves, contaminated with pesticide on the inside.

Contamination by deposition may occur when workers are engaged in environments where pesticides are present as aerosols. Aerosols may form during treatment or other operations, such as manipulation of leaves or other material containing pesticide residues.

Skin contamination may also occur by contact with surfaces bearing pesticide residues. Transfer from surfaces to the skin is a complex process influenced by factors such as contact pressure, affinity of the substance for the skin surface, working methods and hygiene. Contact is the made source of exposure of farm workers re-entering a sprayed area.

Skin exposure may contribute to exposure by other routes. Residues on the

hands can be transferred to the eyes, nose and mouth and may contaminate food, cigarettes and drinks. Hand contact with other parts of the body may spread the contaminant to the genitals. Residues on skin and clothes may be a source of para-occupational exposure (other family members).

Evaluation of skin exposure, difficult to predict a priori, is crucial for identifying sources and mechanisms of contamination, as well as assessing the effectiveness of protective clothing. Since more than 50% of the dose of pesticide may be absorbed through the skin under normal working conditions, evaluation of respiratory exposure alone may not be exhaustive. It is advisable to measure skin exposure and perform environmental sampling and biological monitoring at the same time and evaluate the results as a whole to ensure accuracy in estimates of risk. Measurements of skin contamination are particularly appropriate because few biological indicators of exposure validated for humans are available.

The ideal method of evaluating skin exposure should:

– enable measurement of the quantity of substance available through skin penetration;
– enable an accurate estimate of contamination throughout exposure and sampling;
– enable repetitive sampling in time;
– be applicable to areas of the body regarded as at risk for skin absorption;
– simulate the various processes of skin contamination and removal.

The most widely used methods are discussed below.

3.1. Skin surrogates for evaluating skin exposure

These methods involve placing sampling substrates on the skin and later analysing them to determine pesticide content. The systems used tend to retain substances with low vapour pressure in solid particulate or mist form. The assumption is that the substrate has a similar behaviour to skin, though none of the systems proposed has actually been systematically tested to evaluate retention efficiency. This technique presumably gives overestimates of exposure because the substrates are selected on the basis of their absorbing properties.

3.1.1. *Pads*

Pads cover a small part of the skin area to sample and exposure is calculated by extrapolation of contaminant levels to the whole anatomical district represented. The validity of pads for monitoring exposure depends on various factors. In the case of uniform distribution of the active ingredient on the area of skin, pads provide representative data. Non uniform distribution has been documented in several studies (Fenske, 1990). In these cases pads may lead to over- or under-estimation of real skin exposure.

Although the pad technique is not always accurate for estimating cutaneous dose, it is widely used because it is cheap and easy to perform. Table 5 shows some of the studies reported in the literature in which skin exposure was evaluated by this method.

Table 5. Use of pads of various materials to evaluate skin exposure.

Pesticide	Pad material	Task	References
2,4-D, MCPA	α-cellulose	Treating cereals	Aprea et al., 1995
Acephate	Surgical gauze	Formulation	Maroni et al., 1990
Chlorpyrifos	Surgical gauze	Treating buildings	Fenske and Elkner, 1990
Chlorpyrifos, Carbaryl, Permethrin	Surgical gauze	Mixing and loading	Byers et al., 1992
Clorothalonil	Surgical gauze	Mechanical harvest of tomatoes	Spencer et al., 1991
Deltamethrin, Fenvalerate	Surgical gauze	Treating cotton	Chen et al., 1991
Deltamethrin, Fenvalerate	Surgical gauze	Treating cotton	Zhang et al., 1991
Dimethoate, Mancozeb/ETU	α-cellulose	Formulation	Aprea et al., 1998
EBDC-ETU	α-cellulose	Treating potatoes	Kurttio and Savolainen, 1990; Kurttio et al., 1990
Fluvalinate, Dicofol, Chlorpyrifos, Ethazol	α-cellulose	Treatment in greenhouse	Stamper et al., 1989b
Fosethyl-Al	Surgical gauze	Treating ornamental plants in greenhouse	Fenske et al., 1987
Glyphosate	Surgical gauze	Work in conifer nursery	Lavy et al., 1992
Metomyl, Carbendazim, Captan, Endosulfan, Fenarimol, Pirazophos	Glass-fiber	Treatment in greenhouse	Stephanou and Zourari, 1989
Mevinphos	α-cellulose	Treatment and re-entry of greenhouse	Kangas et al., 1993
Omethoate, Fenitrothion	α-cellulose	Re-entry of greenhouse	Aprea et al., 1994a; Aprea et al., 1998
Pirimiphos-methyl, Dimethoate, Permethrin	Surgical gauze	Tomato harvest	Adamis et al., 1985
Alachlor, Metolachlor, 2,4-D, 2,4-D-2-butoxyethyl ester, 2,4-D-2-ethylhexyl ester, Atrazine, Cyanazine, Simazine	Polyurethane foam	–	NIOSH, 1998c

Materials. The choice of material for pads is problematical because of large variations between and within individuals (dry, damp, hairy, smooth, rough, callous skin etc.) that makes it difficult to define standard skin and thus choose a synthetic substitute.

Two types of material are generally used, alpha-cellulose for exposure to liquids (Aprea et al., 1994a; Aprea et al., 1995; Aprea et al., 1998; Kurttio and Savolainen, 1990; Stamper et al., 1989b; Kangas et al., 1993) and surgical gauze for dry powders and granular materials or when good mechanical resistance is required (Maroni et al., 1990; Fenske et al., 1987; Fenske and Elkner, 1990; Byers et al., 1992; Spencer et al., 1991; Chen et al., 1991; Zhang et al., 1991; Lavy et al., 1992; Adamis et al., 1985). Some authors use other materials, such as glass fibers (Stephanou and Zourari, 1989) and polyurethane foam (NIOSH, 1998c).

EPA recommends that pads of alpha-cellulose be of paper pulp or similar material,

about 1 mm thick (EPA, 1987a). Nevertheless, many different types of cellulose have been used, such as filter paper of different types, preparatory chromatography paper, and so forth. Pads consisting of various layers of surgical gauze are not necessarily sterile and have a backing of filter paper, glass fiber, aluminium foil or plastics. Laboratory tests have shown that gauze pads retain about 90% of powder applied to them, even if inverted or shaken (Durham and Wolfe, 1962).

Other materials include certain types of fabric and plastics: cotton pads circling the arms and legs (Bandara et al., 1985; Winterlin et al., 1984) have been used to monitor exposure to paraquat and captan; synthetic materials such as polyester have also been used (Knaak et al., 1978). A thin transparent film of polyethylene was used for carbofuran (Hussain et al., 1990) based on preliminary tests that demonstrated that more than 98% of the active ingredient adhered to the pad in 5 h.

Fabric pads have problems of standardisation: the type of manufacture, thickness, pretreatments and finishing operations may modify adsorption, retention and permeation of active ingredients. Since manufacturing characteristics vary widely, comparison of data obtained in different studies is difficult. Even washing, which may remove finishing materials, may affect retention. An advantage of fabrics is their easy use because they are easily put in place.

Aluminium foil has been used for oil formulations (WHO, 1986a). Pads impregnated with dense liquids have been used to increase retention capacity (Carman et al., 1982; Grover et al., 1986a; Grover et al., 1986b): sampling efficiency of parathion and dimethoate on gauze pads improved after immersion for 10 min in a 10% solution of ethylene glycol in acetone (Carman et al., 1982). A similar improvement was used with fiberglass pads for sampling the ammonium salt of 2,4-dichlorophenoxyacetic acid (2,4-D) (Grover et al., 1986a; Grover et al., 1986b). This approach seems promising for increasing the specificity of pads as a sampling device.

It is not yet clear whether pads should be extracted with a volume of solvent equal to that utilised for analysis before they are used. In general, this step can be considered if there are interfering compounds.

Pads are generally backed with some other material which may be plastic, fiberglass, aluminium foil or multiple layers of filter paper. Fiberglass support is often used in EPA studies. More than one type of backing is a possibility (Kamble et al., 1992). Backing is used to avoid contact of the sampling substrate with the sweat and oil of the skin. Sometimes the pad is mounted in a frame that leaves the sampling surface exposed (NIOSH, 1998c).

Position. The site where pads are placed depends on sampling strategy: if evaluation of skin exposure only regards exposed skin, the site will depend on the type of protective clothing worn. If, on the other hand, the aim is to evaluate contamination on the whole skin surface, pads will be placed all over the body, even under protective clothing. Many authors (Adamis et al., 1985; Aprea et al., 1994a; Aprea et al., 1998; Brouwer et al., 1993; Byers et al., 1992; Fenske and Elkner, 1990; Kangas et al., 1993; Kurttio and Savolainen, 1990; Kurttio et al., 1990; Lavy et al., 1992; Stamper et al., 1989; Zhang et al., 1991) use this second approach.

More in detail, four approaches are possible:

a) measure potential dermal dose, placing pads in top of clothing;
b) measure actual dermal dose, placing pads in contact with skin under clothing;
c) measure potential and actual dermal dose, placing pads in contact with the skin and on top of clothing;
d) measure protection afforded by clothing, placing pads in contact with skin and under and on top of protective clothing.

Table 6 shows positioning criteria proposed by Davis (Davis, 1980) and Aprea (Aprea et al., 2001a).

With regard to exposure of the head, which is the part most often exposed, it has been proposed to apply pads directly to the skin of the face, forehead or neck (Aprea et al., 1998; Aprea et al., 1999b; Maroni et al., 1990) or to use results obtained with pads on the chest and shoulders (Byers et al., 1992; Fenske et al., 1987; Fenske and Elkner, 1990).

EPA proposes two procedures of pad location (EPA, 1987a). If workers do not wear protective clothing, it is recommended to position at least 10 pads: posterior arms between wrist and elbow, upper back just under collar, upper chest near jugular vein, right and left shoulders, anterior legs under the knee, anterior thighs. If protective clothing is worn, six pads are sufficient as leg pads are unnecessary.

The procedure proposed by the World Health Organisation (WHO, 1986a), widely used in European studies, recommends placing pads on top of protective clothing, if worn, otherwise on the skin. The positions recommended are: left arm between elbow and wrist, anterior left leg below hip, anterior left leg at mid thigh, sternum, between shoulders and forehead (on the right for left-handed subjects). Another pad is placed on the skin of the upper abdomen.

Size. In most cases, the percentage of skin covered by pads is low. In many studies, it has been about 8% which is higher than that suggested by the WHO protocol, namely 3% (Chester, 1993).

Table 6. Selection of body sites for pads and skin areas represented.

Aprea et al., 2001a		Davis, 1980	
Pad Llocation	Skin area represented	Pad location	Skin area represented
face	head and neck	shoulders	head
anterior chest	anterior shoulders and chest	back	back and posterior neck
posterior chest	posterior shoulders and back	chest	anterior neck, chest and stomach
right arm	arms	shoulders and forearms	arm
left forearm	forearms	forearms	forearm
left anterior thigh	anterior thighs and hips	thigh	thigh
right posterior thigh	posterior thighs and hips	ankle	leg
left calf	calves		
right shin	shins and feet		

Pad area varies, generally being 100 cm^2 or more (Kangas et al., 1993; Kurttio et al., 1990; Llewellyn et al., 1996; Stamper et al., 1989a). Other authors have used pads measuring 41–79 cm^2 (Adamis et al., 1985; Aprea et al., 1994a; Aprea et al., 1998; Aprea et al., 1999b; Byers et al., 1992; Fenske et al., 1987) with smaller sizes (<30 cm^2) being used for the face (Aprea et al., 1998; Zhang et al., 1991) where space to apply them is less than elsewhere on the body. NIOSH (NIOSH, 1998c) recommends 10 cm square pads (100 cm^2) in a holder with a circular hole of diameter 7.6 cm on one side.

Calculation of hourly dermal exposure. To calculate exposure of the various skin areas, the concentration of pesticide per unit surface of pad (Ci) is multiplied by the surface area of the anatomical district represented (Si). The sum of exposures obtained for the various areas of the body divided by the time of exposure (T) in hours gives hourly dermal exposure (HDE):

$$\text{HDE} = \frac{\sum_{i=1}^{n} (Ci \cdot Si)}{T}$$

To calculate the surface area of different parts of the body, there are various models (Table 7). The most widely used is the anatomical model (Popendorf and Leffingwell, 1982) and its variations (Adamis et al., 1985; Llewellyn et al., 1996).

Table 7. Methods used to estimate surface area of various anatomical districts of the human body, assuming a total skin area of 1.9 m^2 (Popendorf and Leffingwell, 1982).

Part of body	Wiedenfeld (Berkow, 1931)[a]	Berkow, 1931	Cylindrical model	Anatomical model
Head	4.8	6	9.7[b]	5.7
Neck	2.1		1.1	1.2
Arms	10	13.5	7.0	9.7
Forearms	7.1		9.8[c]	6.7
Hands	4.2	4.5		6.9
Fingers			3.3	
Shoulders				6.8
Chest	27	38[d]		8.0
Back			30.8	8.0
Hips	25	17	20.9	9.1
Thighs				18.0
Calves	12.5	12.7	17.4[c]	13.5
Feet	7.1	6.3		6.4

[a] As reported by Berkow (Berkow, 1931); [b] the model assumes the head to be cylindrical; [c] the hands and feet are included with forearms and calves; [d] the proportions of the Berkow model (Berkow, 1931) can easily be compared with other models if the percentage attributed to the trunk is halved.

Total body area (TBA) can be calculated using various formulae, including those proposed by Du Bois (Du Bois and Du Bois, 1916):

$$\text{SCT (cm}^2) = 71.84 \cdot \text{weight (kg)}^{0.425} \cdot \text{length (cm)}^{0.725}$$

Even when TBA values are obtained, it is not immediate to calculate absorbed dose because it is necessary to know the penetration of the substance across the skin barrier (Sartorelli et al., 1997). Some researchers have used a dermal absorption of 10% (Brouwer et al., 1992b; Brouwer et al., 1992c; Byers et al., 1992) and others (Feldman and Maibach, 1974a) report specific absorptions for various pesticides ranging from 5% to 20%, obtained on the basis of urinary excretion of metabolites within 120 h of administration to volunteers. In other studies, absorptions of 3% have been documented for chlorpyrifos (Fenske and Elkner, 1990) on the basis of studies with volunteers (Nolan et al., 1984).

3.1.2. *Clothing*

Clothes as skin surrogates cover whole skin districts or even the whole body. In the latter case no extrapolation is needed because the levels determined are for the whole area considered. Unlike pads, this method does not require uniform distribution of the pesticide on the area of skin in question. In theory, it may be applied to all parts of the body in different types of occupational activities. Table 8 shows past studies in which skin exposure was evaluated analysing clothes.

Garments covering the whole body surface (whole body garment samplers) such as overalls with hood are used. After exposure they are removed with care and divided into parts matching the various skin districts, which are analysed separately (Abbott et al., 1987; Chester et al., 1987; Guidotti et al., 1994; Spencer et al., 1991). A preliminary choice of overall material is fundamental since it has been shown, for example, that a compound such as ethazol penetrates Tyvek (Stamper et al., 1989c). To evaluate exposure during pesticide dispersal, WHO (1982a) recommends clothing that completely covers the body: workers are required to wear a new overall for at least an hour on days when they are engaged in spraying. In calculating total potential exposure, if face contamination is not evaluated in some way (e.g. by pads or hat), the measure obtained for the overalls should be increased by 10% (WHO, 1982a).

In other studies, clothes that only covered part of the body, such as gloves (Adamis et al., 1985; Brouwer et al., 1992a; Brouwer et al., 1992b; Brouwer et al., 1992c; Byers et al., 1992; Llewellyn et al., 1996), have been used. Gloves are often used to estimate hand exposure during harvest of fruit and vegetables or flowers treated with pesticides. Other garments include t-shirts (McCurdy et al., 1994; Sell and Maitlen, 1983; Ware et al., 1974) and socks (Abbott et al., 1987; McCurdy et al., 1994).

Cotton and nylon gloves have different adsorption with respect to skin and may hinder manual dexterity. Hands vary considerably in size and shape, making it difficult to find gloves that suit everybody. Glove material may contain substances

Table 8. Use of clothes to evaluate skin exposure.

Pesticide	Type of clothes	Task	Reference
2,4-D	Overalls with hood, t-shirt and socks	Treatment of forests	Abbott et al., 1987
2,4-D, Trifluralin, Carbofuran	Overalls with hood, t-shirt and socks	Recycling containers	Guidotti et al., 1994
Azinphos-methyl	Overalls with hood, t-shirt and socks	Peach harvest	McCurdy et al., 1994
Cipermethrin	Overalls with hood, t-shirt and socks	Aerial spraying of cotton	Chester et al., 1987
Chlorothalonil	Overalls with hood, t-shirt and socks	Mechanical harvest of tomatoes	Spencer et al., 1991
Abamectin, Dodemorf, Bupyrimate	Cotton gloves	Greenhouse re-entry	Brouwer et al., 1992c
Chlorpyrifos, Carbaryl, Permethrin	Cotton gloves	Mixing and loading	Byers et al., 1992
Chlorothalonil, Tiophanate-methyl, Thiram, Zineb	Cotton gloves	Greenhouse Cultivation of carnations	Brouwer et al., 1992b; Brouwer et al., 1992c
Permethrin	Cotton gloves	Public hygiene	Llewellyn et al., 1996
Pirimiphos-methyl, Dimethoate, Permethrin	Cotton gloves	Tomato harvest	Adamis et al., 1985

that interfere with analysis, especially when it is necessary to detect microcontamination. Pre-extraction may only partially eliminate interfering compounds. Gloves absorb moisture which may lead to hydrolysis of pesticide. Other materials (e.g. oil residues, fruit juices released during harvest) may be absorbed by gloves and other clothing, causing analytical interference.

To evaluate hand contamination during re-entry of treated crops, EPA recommends use of adsorbing gloves (EPA, 1987a). Although short gloves have mostly been used, long gloves make it possible to estimate exposure of wrists and forearms. Gloves used as sampling substrates can be worn as protective clothing or under protective gloves to evaluate their permeability. In one study (Bandara et al., 1985) leather gloves were used as sampling substrate for paraquat, being worn over rubber gloves which were used for extra protection in case the pesticide permeated the leather gloves.

As described for hands, cotton or nylon socks can be used to measure the quantity of pesticide penetrating shoes (Wolfe et al., 1961). However, sweating may interfere with the measurement, reducing adsorption efficiency considerably. If shoe material permits, internal washing of shoes could be more useful than sock analysis. Shoes of disposable material have been used to evaluate exposure to paraquat during harvest of treated plants (Bandara et al., 1985).

To evaluate skin exposure through the face and scalp during dispersal of 2,4-D, paper hats have been tested (Taskar et al., 1982). The results showed that exposure through the head was greater than that through the chest and back.

Skin contamination of the face can be evaluated by determining the quantity

of active principle deposited on the respiratory mask. In this case, the sampling area is greater than that of the face, so the estimate of dermal exposure is meaningful.

Trousers of different material have been used to monitor skin contamination on the legs. Garments such as blue jeans have been analysed to determine parathion and parathion-methyl during re-entry of treated fields (Ware et al., 1974).

When clothing is used for sampling, saturation of the garment must be avoided. Exposure may vary considerably from one part of the body to another and double layers of clothes may be necessary for parts with high potential contamination, when pesticide could pass through the outer clothing.

Clothing may be a nuisance to workers and cause excessive sweating. For some jobs, clothing may be subject to tearing and may need to be replaced during the work shift. A substantial disadvantage is the difficulty of standardising the material used (type of fibre, thickness, weight, etc.) so that results can be compared with those of other studies.

3.2. Removal techniques for evaluating skin exposure

These techniques are based on measurement of the amount of substance that can be removed from the skin at the time of sampling. It rarely indicates total skin contamination incurred during work. Removal may be done by washing or wiping. Washing is mostly used for the hands, whereas wiping can be applied to the whole body surface and is done with filters, gauze and other pre-moistened commercial materials.

Wipe tests give results that vary in relation to how they are done and are therefore not optimal for evaluating skin contamination by pesticides. Moistened tissues have been used to monitor face and hand contamination in workers harvesting peaches treated with azinphos-methyl (McCurdy et al., 1994). A problem associated with this technique is how to measure the area of skin monitored. The problem is avoided by wiping a well defined area, for example the palm of the hand, which is sampled separately from the fingers.

Wipe tests are more widely used to evaluate contamination of surfaces (floors, walls, furniture and so forth) in interiors (offices, dormitories, etc.) treated for public hygiene (Currie et al., 1990; Wright et al., 1993). They are not suitable for volatile substances, because much of the pesticide is lost by evaporation before analysis. The main substrates are filters, gauze, cotton wool moistened with isopropanol or other solvents.

3.2.1. *Hand washing*

EPA recommends hand washing for evaluation of exposure to pesticides (EPA, 1987a). This technique is indicated for substances that are not readily absorbed through the skin, and unsuitable for organophosphorus insecticides, unless combined with other sampling procedures, such as garment samplers and biological monitoring (Popendorf and Leffingwell, 1982).

Various solvents and solutions have been used in relation to the solubility of

pesticides to be sampled. Table 9 lists hand wash liquids most widely used for different applications.

It is possible to standardise washing techniques and certain authors have proposed a procedure to evaluate the efficiency of removal (Fenske and Lu, 1994). Removal decreases with decreasing exposure and with increasing interval between contamination and sampling.

In the original technique of Durham and Wolfe (1962), one hand was washed at a time by immersing it in a polyethylene bag containing 200 ml solvent consisting of ethanol or water (bag method). The hand and bag were shaken vigorously and repeated one or more times. Before use, the bag was pretreated with the sampling solvent to check for interfering substances. The thickness of the material of the bags needs to be at least 0.025 mm to ensure sufficient strength (Davis, 1980). The bag method has been used by various authors, even recently (Fenske et al., 1987; Fenske and Elkner, 1990; Verberk et al., 1990). NIOSH method 9200 (NIOSH, 1998d) also envisages 150 ml isopropanol in a polyethylene bag 0.1 mm thick and measuring 30.5×20.3 cm.

Another hand wash technique involves pouring solvent over one hand at a time or both hands as they are rubbed together (pouring method) (Aprea et al., 1994a;

Table 9. Hand washing to evaluate skin exposure.

Pesticide	Wash liquid	Task	Reference
Dimethoate, Mancozeb/ETU	Ethanol	Formulation	Aprea et al., 1998
Omethoate, Fenitrothion	Ethanol	Re-entry of greenhouse	Aprea et al., 1994a; Aprea et al., 1999b
2,4-D, MCPA	Ethanol	Treatment of cereals	Aprea et al., 1995
Mevinphos	Ethanol	Treatment and re-entry in greenhouse	Kangas et al., 1993
Fluvalinate, Dicofol, Chlorpyrifos, Ethazol	Ethanol	Treatment in greenhouse	Stamper et al., 1989b
Chlorpyrifos-methyl, Azinphos-methyl	Ethanol	Thinning of juvenile fruits	Aprea et al., 1994b
Chlorobenzylate	Ethanol	Orange harvest	Stamper et al., 1986
Acephate	Water	Formulation	Maroni et al., 1990
Chlorotalonil	Water with surfactant	Mechanical harvest of tomatoes	Spencer et al., 1991
Azinphos-methyl	Water with surfactant	Peach harvest	McCurdy et al., 1994
Maneb, Zineb	EDTA	Flower bulb cultivation	Verberk et al., 1990
Glyphosate	Methanol/water	Work in conifer nursery	Lavy et al., 1992
Chlorpyrifos	Isopropanolo/water	Treatment of buildings	Fenske and Elkner, 1990
Fosetil-Al	Isopropanol/water	Treatment of ornamental plants in greenhouse	Fenske et al., 1987
Alachlor, Metolachlor, 2,4-D, 2,4-D-2-butoxyethyl ester, 2,4-D-2-ethylhexyl ester, Atrazine, Cyanazine, Simazine	Isopropanol	–	NIOSH, 1998d

Aprea et al., 1994b; Aprea et al., 1998; Aprea et al., 1999b; Maroni et al., 1990). The liquid is collected in a special container held under the hands. Use of a teflon brush has also been proposed (Maroni et al., 1990). A volume of 250 ml is generally used for each hand (Fenske et al., 1987; Fenske and Elkner, 1990), though volumes from 90 ml per hand to 200 ml for both hands have been proposed (Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 1998; Aprea et al., 1999b; Kangas et al., 1993).

If hand wash methods are used, it is preferable that sampling be carried out as soon as contamination occurs. However, frequent washing can alter the barrier properties of the skin. In most cases, therefore, hand washing is done at the beginning and end of the work shift (Fenske et al., 1987; Fenske and Elkner, 1990) though some authors do 3–4 washes per shift (Aprea et al., 1994b; Kangas et al., 1993).

To evaluate hand contamination, gloves have several advantages over washing: they do not require solvents which may destroy skin lipids, causing irritations that may give rise to higher absorption of active principles (van Hemmen and Brouwer, 1995). Liquids (water and ethanol) can cause breakdown of pesticide residues (van Hemmen and Brouwer, 1995). Gloves, like clothes, may contain interfering substances that need to be removed first. It is also difficult to convince workers to wear gloves for certain tasks.

3.3. Fluorescent tracer technique for evaluation of skin exposure

Skin exposure can be evaluated by measuring deposition of fluorescent material on the skin using video images. Since most pesticides are not naturally fluorescent, a tracer, usually 4-methyl-7-diethylaminocoumarin) must be added to the formula before use. Deposition of tracer on the skin can be evaluated for the whole body surface. The method involves obtaining images of the skin, illuminated with UV radiation, before and after exposure. Once standard curves have been plotted and the concentration ratio of active ingredient to tracer established, the technique can provide quantitative data on skin contamination. However, the method is prevalently used for qualitative studies, also in operator training procedures. The fluorescent tracer method has been used to evaluate non uniform distribution of pesticides on the skin of occupationally exposed subjects (Fenske, 1990; Fenske, 1993). Another application is to determine the best position for pads (Fenske, 1993).

In theory, this method can provide an accurate evaluation of skin exposure since uniform distribution on the body surface is not a necessary condition. It also provides information on exposure of skin surfaces covered or otherwise by work clothes.

The many limits of the technique, especially for quantitative use, include:

– the need to add extraneous substance to the formula. If the pesticides are used in agriculture, this may not be a problem as the tracer is not toxic to plants. On the other hand, the tracer may be incompatible with industrial processes of synthesis and formulation.
– The technique must be properly validated, in particular to detect any breakdown of tracer by sunlight.

– If the workers wear protective clothing, further studies are necessary to evaluate passage of tracer and pesticide through the fabric.

3.4. Determination of removable residues for evaluation of skin exposure

Removal methods have been used to monitor subjects exposed through contact with leaves, flowers and fruit bearing residues of previous treatments. In this situation, estimates of risk mainly regard compounds that can be transferred from the contaminated surface to the skin (dislodgeable residue). To evaluate DR, leaves, flowers etc. are washed with liquids such as water, or aqueous solutions containing NaCl or surfactants. Table 10 lists papers in the literature concerned with determination of DR.

DFR (dislodgeable foliar residues) is usually expressed as mass per unit surface area of leaves. To evaluate the area sampled, some authors (Brouwer et al., 1992c; Goh et al., 1986) measure the area of single leaves. Because this is time-consuming, Goh et al. (1986) proposed regressions between area and fresh weight of leaves.

If possible, punches of a given diameter are used to obtain leaf discs which are collected in a glass container which can be sealed and stored. The total area sampled is obtained multiplying the number of discs by disc area. Clearly this method cannot be used for very small leaves such as carnation leaves or grass

Table 10. Use of various aqueous solutions to evaluate dislodgeable residues.

Pesticide	Aqueous wash solution	Type of surface washed	Reference
Chlorpyrifos, dichlorvos	sur-ten* or triton-x100 or other surfactants	Grass	Goh et al., 1986
Propoxur	sur-ten* or triton-x100 or other surfactants	Ornamental plants	Brouwer et al., 1993
Chlorothalonil	sur-ten* or triton-x100 or other surfactants	Tomatoes	Spencer et al., 1991
Azinphos-methyl	sur-ten* or triton-x100 or other surfactants	Peach leaves	McCurdy et al., 1994
Chlorothalonil, thiram, tiophanate-methyl	sur-ten* or triton-x100 or other surfactants	Carnations	Brouwer et al., 1992b
Chlorobenzilate	sur-ten* or triton-x100 or other surfactants	Oranges and leaves	Stamper et al., 1986
Bendiocarb	sur-ten* or triton-x100 or other surfactants	Azaleas	Nigg et al., 1992
Propargite	sur-ten* or triton-x100 or other surfactants	Peach leaves	Smith, 1991
Organophosphorus insecticides	20% w/v NaCl		Berck et al., 1981
Mevinphos	water	Ornamental plants	Kangas et al., 1993
Glyphosate	water	Conifers	Lavy et al., 1992
Azinphos-methyl, Phosmet, carbaryl	water	Peach leaves	Bowman et al., 1982

(Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 1999b; Kangas et al., 1993).

Sampling must be representative. Fully developed leaves are preferred because residues may undergo dilution of active ingredient in time on juvenile leaves. The discs should be punched from the centre of the leaf (Iwata et al., 1977).

If measured over a period of time, the half-life of pesticides can be calculated from DFR, usually by means of a log-linear type model (Smith, 1991). A statistically significant correlation has been found between DFR and pesticide aerosol levels released during movement of leaves (Aprea et al., 1999b).

If appropriately validated, the DFR technique is promising for quantitative studies. Since the whole surface considered (leaves, flowers, fruit) is washed, the question of representativity of the sample area does not arise, unlike for wipe tests.

3.4.1. *Dermal transfer coefficient*

If sampling of contaminated surfaces and skin are carried out at the same time, it is possible to calculate the dermal transfer coefficient (DTC) for a given occupational activity. Dermal exposure can subsequently be estimated from DFR values of contaminated surfaces. DTC expresses the frequency of contact per unit area and is the ratio of dermal exposure DE to DFR. The general formula is:

$$DTC = \frac{DE(mass/h)}{DFR(mass/cm^2)}$$

These coefficients have been determined for a certain number of occupational situations in agriculture (Krieger et al., 1990) and for example during harvesting of tomatoes, oranges and other fruit such as grapes and strawberries (Spencer et al., 1991; Stamper et al., 1986).

DTC values make it possible to classify single tasks in agriculture and the pesticides used for all types of crop. This classification enables ranking of risk in relation to the degree of pesticide contamination possible by contact, leading to useful information on PIE (protective individual equipment), especially choice of gloves.

4. RISK EVALUATION

4.1. Comparison of doses with dermal LD50

As reported by Durham and Wolfe (1962), occupational exposure may be calculated as percentage of toxic dose per hour (PTDPH) according to the formula:

$$PTDPH = \frac{DE + RE \cdot 10}{LD_{50}D \cdot b.w.}$$

where DE is dermal exposure in mg/h, RE respiratory exposure in mg/h and $LD_{50}D$ dermal LD_{50} in mg/kg body weight multiplied by body weight (b.w.). A factor of 10 was used empirically for respiratory absorption which is faster and more complete than dermal absorption. Using this type of calculation, some authors (Adamis et al., 1985; Byers et al., 1992; Wolfe et al., 1972) claim that acute poisoning can be avoided if exposure does not exceed 1% of the toxic dose (PTDPH ≤ 1%).

4.2. Comparison of doses with no observed effect levels (NOELs)

Acceptable risk is evaluated by many authors (Aprea et al., 1998; Byers et al., 1992; Franklin et al., 1986) by comparing exposure data and no observed effect levels (NOEL), if available.

Byers et al. (1992) introduced the margin of safety (MOS):

$$MOS = \frac{NOEL}{AD}$$

where AD (absorbed dose) (mg/kg/day) is the sum of dermal and respiratory doses. To obtain the dose absorbed through the skin, the authors used a skin penetration value of 10%.

Other authors (Brouwer et al., 1992a; Brouwer et al., 1992b) used NOEL to establish respiratory and cutaneous indicative limit values (ILV) which represent the highest mean level of exposure that does not adversely affect health. To establish these limits (mg/day), NOEL (mg/kg b.w.) is multiplied by b.w. and corrected for absorbed fraction (AF) with a safety factor (SF). The model is:

$$ILV = \frac{NOEL \cdot b.w.}{A.F. \cdot S.F.}$$

The absorbed fraction is generally taken to be one when calculating respiratory ILV and 0.1 for cutaneous ILV. The safety factors differ from substance to substance and are used for intra and interspecies differences in the absence of gene toxic and reproductive effects.

Dermal ILVs have been calculated for dodemorf, abamectin and bupyrimate (Brouwer et al., 1992a). Respiratory ILV have been calculated for chlorthalonil, thiophanate-methyl, thiram and zineb (Brouwer et al., 1992b).

The problems encountered comparing estimated dose with NOEL depend on three main factors:

– NOEL is generally obtained through studies with animals rather than humans;
– NOEL refers to oral dose whereas occupational exposure is prevalently dermal and only partly respiratory;
– there have been few studies to evaluate dermal and respiratory absorption under real conditions of pesticide use.

4.3. Comparison of doses with acceptable daily intake (ADI)

Some authors (Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 1999b; Aprea et al., 2001a; Aprea et al., 2002) have compared exposure data and acceptable daily intake (ADI), or the quantity of pesticide that can be absorbed daily for a lifetime without manifesting toxic effects. Although ADI is calculated for the general population, which is exposed to pesticides prevalently through food, it is a widely used reference, below which occupational risk is probably negligible.

5. BIOLOGICAL MONITORING

The best way to acquire knowledge of exposure levels is by measuring the dose which has entered the organism, and this can be mainly done through biological monitoring. In some cases, where exposure levels fluctuate over time, and/or the skin represents a significant route of absorption into the organism, biological monitoring has proven to be a reliable tool for collecting information on the absorbed dose.

Biological indicators currently available for monitoring pesticide exposure in man can be divided into three main groups: biological indicators of dose or exposure, biological indicators of effect and biological indicators of effective dose. The term 'biological indicator of dose' means the measurement and assessment of chemical agents (or their metabolites) either in tissues, secreta, excreta, exhaled air, or any combination of these in order to evaluate exposure and health risk and compare them with an appropriate reference (Berlin et al., 1984). Pesticides not, or relatively little, transformed by the body can be determined as such in biological liquids. These measurements are highly specific and possible for cyclopentadiene organochlorines (aldrin, dieldrin) (WHO, 1989), cycloparafins (lindane) (WHO, 1982b), phenylparafins (DDT) (Coye et al., 1986a), dipyridyl derivatives (paraquat, diquat) (WHO, 1984b) and derivatives of phenoxycarboxylic acid (2,4-D, MCPA) (Aprea et al., 1995; Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b; Lavy and Mattice, 1986; WHO, 1984a). For most other compounds, metabolites of different specificity are used to indicate dose or exposure (Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 1996a; Aprea et al., 1996b; Aprea et al., 1998; Boleij et al., 1991; Brouwer et al., 1993; Chester et al., 1987; Coye et al., 1986a; de Cock et al., 1995; Fenske and Elkner, 1990; Franklin et al., 1986; He et al., 1988; Huang et al., 1989; Kurttio et al., 1990; Llewellyn et al., 1996; Verberk et al., 1990; Wang et al., 1987; WHO, 1982a; WHO, 1986b; WHO, 1988; Wollen, 1993; Zhang et al., 1991).

In some cases it is possible to measure early changes attributable to exposure. If these changes are 'non adverse' and reversible, and if a dose/effect relationship were known, these changes could be used for biological monitoring of exposure as biological indicators of effect.

In other cases it is possible to measure the product of the linkage of the chemical under study, or its metabolites, to specific cellular receptors. When available, these indicators are the so-called 'biological indicators of effective dose'.

Despite the importance of this problem, biological monitoring of pesticide exposure is not yet carried out on a routine basis in field activities. Briefly, the reasons are:

1. Analytical methods currently available are often very complicated and imply laborious preparation of samples followed by sophisticated analysis involving, for example, chromatography or mass spectrometry. This means that analysis can only be done in a few highly specialized laboratories.
2. Pure commercial standards for metabolites are lacking.
3. Very few completely validated methods exist that are recommended by reference organizations.
4. In field studies of pesticide exposure it is difficult to establish a sound sampling strategy with representative samples and a correct sampling period.
5. Permissible exposure limits and biological exposure indexes are only available for a limited group of compounds. The lack of biological limits is partially compensated by good number of reference values, namely indicator concentrations typically measured in the general 'unexposed' population. Unfortunately, these values only indicate the extent of exposure but do not provide the necessary information for estimating health risk.

Biological monitoring is not appropriate if the pesticide is metabolised into many minor metabolites. Ideally, a metabolite can be used if it represents 30% of the dose absorbed (Wollen, 1993). However, depending on the risk to assess, it may sometimes be possible to use a minor metabolite as biological indicator of exposure in the absence of major biological markers.

To ensure reliable quantitative data on pesticides absorbed occupationally, it is necessary to know something about their metabolism and toxicokinetics, preferably in humans. Results obtained with human volunteers are useful for choosing the biological matrix and methods of sampling. If volunteers cannot be used for ethical reasons, one can resort to studies with experimental animals, though it is not clear to what extent the results can be applied to humans.

Table 11 shows substances for which biological monitoring has been proposed to evaluate occupational exposure.

5.1. Method of sampling and storing biological monitoring samples

5.1.1. *Blood*

In occupationally exposed subjects, blood samples for assay of biological indicators should be obtained at the end of exposure. Since the tissues of persons not occupationally exposed show traces of various compounds (e.g. organochlorines), it is advisable to take pre-exposure blood samples with which to compare the results obtained after exposure. For pentachlorophenol and dinitro-o-cresol, ACGIH (ACGIH, 2002) and WHO (WHO, 1982b) recommend blood sampling at the end of the work shift.

Additional considerations can be made for measurements of cholinesterase activity, which varies widely from person to person. It is therefore advisable that

Table 11. Biological monitoring of occupational exposure to pesticides.

Insecticides	Matrix	Substances analysed	References
Organophosphorus ChE inhibitors	blood	AChE	Coye et al., 1986b; WHO, 1986b
Alkylphosphates	urine	DMP, DMTP, DMDTP, DEP, DETP, DEDTP	Aprea et al., 1998; Coye et al., 1986a; Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 1996a; Aprea et al., 1996b; Aprea et al., 2000; Franklin et al., 1986; WHO, 1986b
Chlorpyrifos Chlorpyrifos-methyl	urine	3,5,6-trichloro-2-pyridinol	Fenske and Elkner, 1990; Aprea et al., 1999a
Chlorpyrifos DEF	blood lymphocytes	neurotoxic esterase (NTE)	Lotti et al., 1983; Lotti, 1986
Acephate	urine	acephate, methamidophos	Maroni et al., 1990
Malathion	urine	mono and dicarboxylic acids	Coye et al., 1986a; WHO, 1982a; WHO, 1986b
Fenitrothion	urine	3-methyl-4-nitro phenol	Liska et al., 1982
Parathion Parathion-methyl	urine	p-nitrophenol	Gallo and Lawryk, 1991; Kummer and van Sitter, 1986
Carbamates ChE inhibitors	blood	AChE	Coye et al., 1986b; Huang et al., 1989; WHO, 1986c
Benomyl	urine	benomyl, carbendazim, MHBC	Liesivuori and Jaaskelainen, 1984
Carbaryl	blood urine	1-naphthol	WHO, 1982a
Carbofuran	urine	3-hydrocaboxyfuran	Huang et al., 1989
Pirimicarb	urine	M1 and M2	Verberk et al., 1990
Propoxur	urine	2-isopropoxyphenol	Brouwer et al., 1993
Syntetic pyrethroids Cypermethrin	urine	DCVA, 3-PBA, 4-OH-3PBA	Chester et al., 1987
Cyfluthrin Deltamethrin	urine urine	F-PBA deltamethrin, DBVA	Zhang et al., 1991; He et al., 1988
Fenvalerate	urine	fenvalerate, 3-PBA, CPBA	Zhang et al., 1991; He et al., 1988; Aprea et al., 1997b; Lavy et al., 1993
Permethrin Organochlorine compounds	urine	permethrin, DCVA, 3-PBA	Llewellyn et al., 1996
Aldrin, Dieldrin	blood	aldrin, dieldrin	WHO, 1989; Tordoir and van Sitter, 1994
Chlordane	blood	trans-nonachlor, heptachlor epoxide, oxychlordane	Saito et al., 1986
DDT	blood urine	DDT/2,2-bis(4-chlorophenyl)-acetic acid	Coye et al., 1986a

Table 11. Continued.

Insecticides	Matrix	Substances analysed	References
1,3-Dichloropropene	urine	cis and trans-DCP-MA	Verberk et al., 1990; Brouwer et al., 1991a; Brouwer et al., 1991b; Brouwer et al., 2000
Endrin	urine	anti-12-hydroxyendrin	Kummer and van Sitter, 1986
Heptachlor	blood	heptachlor epoxide	Mussalo-Rauhamaa et al., 1991
Lindane, HCH	blood	r-HCH, isomers of HCH	WHO, 1982c; Coye et al., 1986a
Herbicides 2,4-D	blood urine	2,4-D	Lavy and Mattice, 1986; WHO, 1984a
MCPA	urine	MCPA	Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b
2,4,5-T	urine	2,4,5-T	Kolmodin-Hedman and Erne, 1980
Alachlor	urine	DEA, HEEA	Wollen, 1993
Fluazifop-butile	urine	fluazifop	Wollen, 1993
Glyphosate	urine	glyphosate	Lavy et al., 1992
Diquat and Paraquat	blood urine	diquat o paraquat	WHO, 1984b
Atrazine	urine	atrazine and dealkylated metabolites	Catenacci et al., 1990; Catenacci et al., 1993
Fungicides Captan	urine	tetrahydrophthalimide	de Cock et al., 1995; de Cock et al., 1998; Krieger and Dinoff, 2000
Maneb Zineb Mancozeb	urine	ethylenethiourea	Boleij et al., 1991; Kurttio and Savolainen, 1990; WHO, 1988; Colosio et al., 2002
Other compounds Chlordimeform	urine	4-chloro-o-toluidine	Wang et al., 1987
Chlorobenzilate	urine	p,p′-dichlorobenzophenone	Stamper et al., 1986
Dinitro-o-cresol	blood	Dinitro-o-cresol	WHO, 1982b; Coye et al., 1986a
Pentachlorophenol	blood urine	Pentachlorophenol	WHO, 1982b; Coye et al., 1986a

DMP (dimethylphosphate); DMTP (dimethylthiophosphate); DMDTP (dimethyldithiophosphate); DEP (diethylphosphate); DETP (diethylthiophosphate); DEDTP (diethyldithiophosphate); DEF (s,s,s-tributyl phosphorotrithioate); M1 (2-dimethylamino-4-hydroxy-5,6-dimethylpyrimidine); M2 (2-methylamino-4-hydroxy-5,6-dimethylpirimidine); DCVA [3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanoic acid]; F-PBA (4-fluoro-3-phenoxybenzoic acid); 3-PBA (3-phenoxybenzoic acid); 4-OH-3-PBA [3-(4-hydroxy)-phenoxybenzoic acid]; DBVA [3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropanoic acid]; HCH (hexachlorocyclohexane); CPBA [2-(4-chlorophenyl)-3-methyl-1 butanoic acid]; MHBC [(methyl (4-hydroxy-1H-benzimidazol-2yl) carbamate], DCP-MA [N-acetyl-S-(3-chloroprop-2-enyl)-cysteine] 2,4-D (2,4-dichlorophenoxyacetic acid); MCPA (2-methyl-4-chlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid); DEA (2,6-diethylaniline); HEEA (2-(1-hydroxyethyl)-6-ethylaniline).

subjects have at least one assessment of both pseudo and true cholinesterase activity before coming into contact with organophosphates or carbamates. These are baseline values that can be compared with post-exposure values to determine the significance of any reduction. WHO recommends three sequential basal samples (WHO, 1982a). After exposure, samples should be obtained within 2 h for organophosphates and as soon as possible for carbamates, due to the rapid reversibility of enzyme inhibition.

5.1.2. *Urine*

A 24-hour urine sample (in a single container or in fractions representing various periods of the day) is generally recommended if the objective is to estimate absorbed dose. Spot urine samples can be obtained at the end of the work shift to determine absorption trends in groups, but are unsuitable for estimating absorbed doses.

More specifically, for biological monitoring of exposure to compounds with slow absorption and excretion (azinphos-methyl, chlorpyrifos, phorate, ethyl-enethiourea, pyrethroid insecticides), it may be necessary to collect urine over 24–48 h from the start of exposure or in some cases a spot sample before the work shift of the day after exposure.

Exposure of farm workers is mainly cutaneous and absorption may be slow and protracted in time. In these cases, a single urine sample at the end of the work shift may not be indicative of absorbed dose. Several studies (Aprea et al., 1994b; Aprea et al., 1997c) have used 24-h urine samples, sometimes divided into several fractions (one during the work shift and one after the shift up to the start of work next day). If exposure extends over several consecutive days, sampling may continue for all working days of the week and for 24–48 h after the last day of work (Aprea et al., 1994a; Aprea et al., 1994b). It is advisable to continue collecting urine for a certain period after exposure; this period should be at least four times the half-life of the substance. This is useful for evaluating elimination kinetics and if possible, absorbed doses.

In any case, and especially if biological monitoring does not begin on the first day of exposure, it is advisable to make a spot urine sample before the work shift (basal sample) (Aprea et al., 1994a; Aprea et al., 1994b; Aprea et al., 2002). Basal samples are important for at least three reasons: even if a worker is not engaged in the task for which biological monitoring is carried out, he nevertheless works on the farm and can have contact with different types of pesticides; the biological indicators used can often be found in urine of subjects not occupationally exposed; in certain cases, for example some metals (copper, manganese, arsenic), the analyte is normally found in the body.

When using spot urine samples, creatinine or specific weight should also be determined in the sample to normalise the results and discard samples which are too dilute or too concentrated. When using 24-h urine samples or fractions representing various intervals of the day, the volume of urine excreted should be determined in order to define the absolute quantities of metabolites present in the sample.

The ACGIH (ACGIH, 2002) recommends urine sampling at the end of the work

shift for assay of p-nitro phenol and before the last work shift of the week for dinitro-o-cresol.

Urine can be collected in plastic containers shielded from the light with aluminium foil. Further considerations on analytical and preanalytical problems regarding biological monitoring of exposure to pesticides may be found in the chapter on the general population in this volume (Aprea, 2003).

5.2. Organophosphorus compounds

Phosphoric esters or organophosphates (OPs) are compounds with a radical containing phosphorus in their molecule. Apart from this basic characteristic, they may be structured in very differentiated ways with aliphatic and/or aromatic groups and/or functional groups containing chlorine and/or nitrogen and/or sulphur.

Hence there are many dozens of OPs that may vary considerably in physico-chemical properties. The toxicological feature common to many of these compounds is that they inhibit the activity of cholinesterase, an enzyme essential for many biological functions, especially that of the central and peripheral nervous systems of humans and animals.

Organophosphorus insecticides may be absorbed by inhalation, ingestion or through the skin. Their chemical nature makes them available for many biotransformations and reactions with tissue constituents, especially tissue proteins with active esterase sites. Biotransformation reactions leading to the disappearance of anticholinesterase activity involve mixed function oxidase, hydrolase and transferase activities (WHO, 1986b).

Organophosphorus insecticides and their metabolites are largely excreted in the urine, with minor quantities eliminated in the feces and expired air. Urinary and fecal elimination is generally rapid with 80–90% of the dose usually eliminated within 48 h, though small quantities may be detected in urine for several days, probably due to storage in fatty tissue and covalent bonds affecting protein phosphorylation (WHO, 1986b). A tiny fraction of OPs and their oxygenated analogues is excreted unmodified in urine. Most of the compounds excreted are hydrolysis products consisting of alkylphosphates and specific phenolic metabolites (Maroni, 1986).

5.2.1. *Blood cholinesterase activity*

Signs and symptoms of organophosphorus and carbamate poisoning are the result of an accumulation of acetylcholine in neuromuscular junctions and other sites of action. Under normal conditions, acetylcholine is hydrolysed to acetic acid and choline by the enzyme acetylcholinesterase (AChE) after transmission of a nerve impulse. There are three classes of esterases: A-esterases are responsible for hydrolysis of organophosphorus insecticides, B-esterases, including acetylcholinesterase, are subject to progressive covalent inhibition by phosphoric esters and carbamates, and C-esterases do not react with these two classes of compound (WHO, 1986b; WHO, 1986c).

Reactivation of the enzyme may occur spontaneously after poisoning at a speed depending on the nature of the group attacked, the type of protein, pH and addition

of nucleophilic agents such as oximes that may act as catalysts and are used to treat cases of acute poisoning (WHO, 1986b).

Although AChE is vital for hydrolysis of acetylcholine and transmission of nerve impulses, other cholinesterases, such as butyrylcholinesterase (BuChE, pseudo-cholinesterase or plasma cholinesterase) do not have any known physiological role and their inhibition is not associated with toxicity of the compound.

Erythrocyte acetylcholinesterase is biochemically identical to the enzyme found in synapses of the central nervous system (target organ) and has been recommended as indicator of effect for biological monitoring of ChE inhibitors (WHO, 1986b). Measured in erythrocytes, it is a better indicator of risk for health than plasma cholinesterase. However, plasma cholinesterase activity is usually more subject to inhibition than true acetylcholinesterase (erythrocyte AChE). After a single dose of organophosphorus insecticide, pseudocholinesterase activity recovers more quickly than that of erythrocytes. After severe poisoning, the reduction in enzyme activity may last as long as 30 days in plasma and 100 days in erythrocytes, which are the periods necessary for the liver to resynthesize pseudocholinesterase and to replace red blood cells (Maroni, 1986).

Inhibition of AChE is usually correlated with the severity of acute poisoning. In the case of chronic or repeated exposure, the correlation with toxic effects may be poor or non existent. Manifestation of symptoms depends more on the speed at which cholinesterase activity drops than on the absolute level reached (Coye, 1986b; Maroni, 1986).

Determination of cholinesterase activity and assay of urinary metabolites of pesticides that inhibit cholinesterases provide complementary information on exposure because excretion of metabolites is fast but enzyme activity recovers slowly. Determination of the latter gives an integration of the effects of exposure over several days, whereas determination of urinary metabolites provides information on very recent exposure (Hayes, 1971; Hayes, 1982).

In healthy subjects, erythrocyte AChE is not affected by physiological factors such as age, sex or race. However, inter and intra-individual variations greater than 13–25% have been detected in subjects not exposed to cholinesterase inhibitors (Coye, 1986b). Because of the wide interval of enzyme activity observed in normal subjects, it is necessary to have pre-exposure values with which to compare post-exposure data. In cases in which pre-exposure activities are not known, mean values of the general population have been used as reference (WHO, 1986b).

Measurements of cholinesterase activity have been widely used in field studies, even to evaluate results induced by changes in work systems, use of PP, distribution systems and to establish intervals for re-entry and so forth. Clinical effects were never observed without large reductions in serum or erythrocyte cholinesterase activity (WHO, 1986b). With regard to interpretation of results, a reduction to 70% of the individual AChE baseline (30% inhibition) has been suggested as an indication of risk of over-exposure. This level is adopted by ACGIH (ACGIH, 2002) and DFG (DFG, 1993) as a biological limit. Since BuChE is more sensitive but less specific, 50% inhibition level has been suggested as a biological limit (WHO, 1982b).

5.2.2. *Neuropathy target esterase (NTE) in peripheral lymphocytes*

Poisoning by certain OPs causes delayed neuropathies in humans, namely polyneuropathy distinguished by acute cholinergic signs, beginning with phosphorylation of a protein of the central nervous system known as neuropathy target esterase (NTE). Inhibition of NTE has been observed in workers exposed to s,s,s-tributyl phophorotrithioate (DEF), a defoliant used on cotton crops, without electrophysiological evidence of effects on the peripheral nervous system (Lotti et al., 1983). This biological indicator has mainly been used in a research setting (Lotti et al., 1983; Lotti, 1986).

5.2.3. *Unchanged compounds*

Acephate and methamidophos. Acephate is metabolised relatively little by the human body, 73–77% of the absorbed dose being excreted unchanged in urine. Most is excreted within 12 h of exposure (Maroni et al., 1990; FAO, 1977). Biological monitoring studies (Maroni et al., 1990) conducted during formulation of acephate have shown peaks of elimination of the compound in urine samples collected during the work shift and in the 8 hours that followed. Elimination was fast and complete within 48 h. Urinary excretion of acephate showed a good correlation with total exposure (cutaneous and respiratory). Although methamidophos was also analysed, this compound was not found in the urine samples collected (Maroni et al., 1990). Acephate in urine may be used as an indicator of exposure but the available data is insufficient to establish exposure limits.

5.2.4. *Metabolites*

Alkylphosphates. Dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate, diethylthiophosphate and diethyldithiophosphate are metabolic products of various OPs. They are formed by hydrolysis of the ester bond in the OP molecule. Dimethyl OPs produce dimethyl metabolites and diethyl OPs produce diethyl metabolites (WHO, 1986b). Alkylphosphates are excreted in urine as sodium or potassium salts. Excretion is usually quite rapid (80–90% of the total dose within 48 h) (WHO, 1986b). Although maximum excretion is usually within 24 h of the start of exposure (Maroni, 1986), it may be useful to prolong urine collection to 48 h after exposure if absorption of OPs is mainly cutaneous.

Alkylphosphates in urine are more sensitive indicators of exposure than acetylcholinesterase inhibition. Unfortunately, biological limits of exposure have not yet been established, and it is complicated to interpret the results in terms of risk for human health. Figure 1 gives mean concentrations of these metabolites found in different occupational situations and in the general population.

3,5,6-Trichloro-2-pyridinol (TCP). TCP is a product of esterase cleavage of chlorpyrifos and chlorpyrifos-methyl (Nolan et al., 1984; Chang et al., 1996). It constitutes 96% of total urinary chlorpyrifos metabolites in rats; 12% is free and the rest conjugated, mainly with glucuronic acid (Sultatos et al., 1982). After oral and dermal
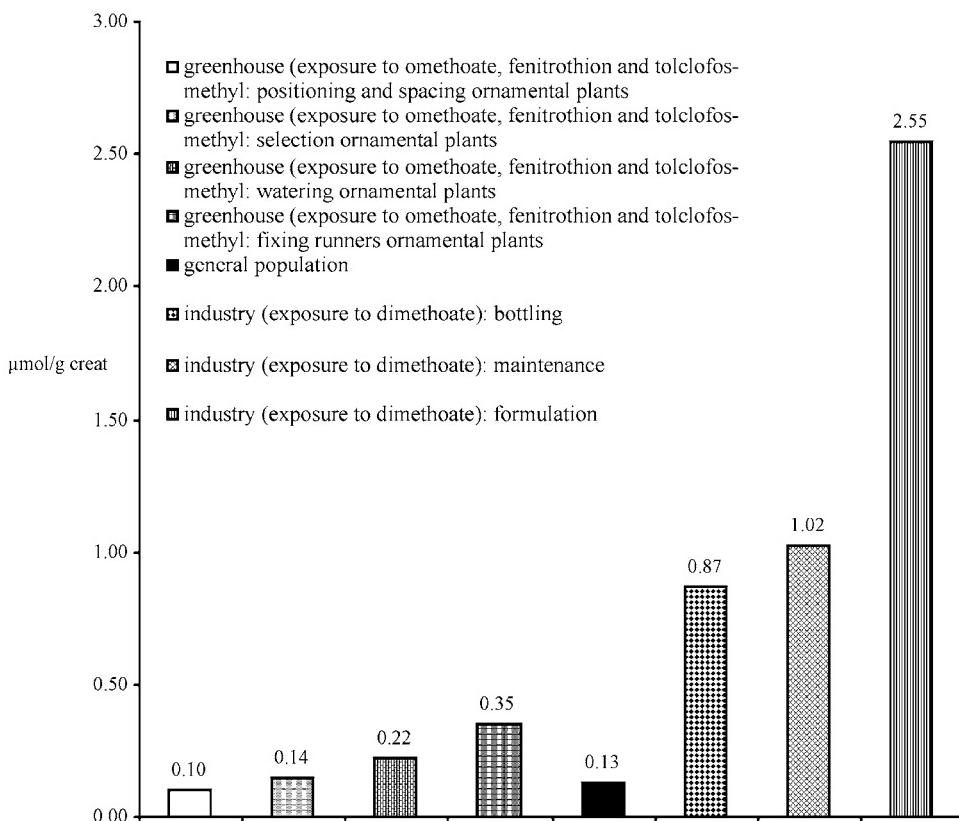
Figure 1. Urinary excretion of alkylphosphates (µmol/g creat) in various tasks (Aprea et al. 1996a, Aprea et al. 1998, Aprea et al. 1999b, Aprea et al., 2001a). Values reported as geometric mean.

administration of chlorpyrifos to volunteers, the biological half-life was found to be 27 h (Sultatos et al., 1982). In humans, about 70% of the oral dose, and less than 3% of the dermal dose, were excreted in urine as TCP (Nolan et al., 1984).

TCP has been assayed in urine of workers exposed to chlorpyrifos (Fenske and Elkner, 1990) and chlorpyrifos-methyl (Aprea et al., 1997a) as well as in the general population (Aprea et al., 1999a; Hill et al., 1995; Kutz et al., 1992). During fumigation of buildings with chlorpyrifos, urine was sampled before exposure and for 72 h after the end of exposure. The best correlation was observed between excretion of TCP in the sample obtained after 24–48 h and the total absorbed dose or dermal dose, which was the prevalent part. Respiratory exposure did not show a significant correlation with excreted metabolites in any period of elimination (Fenske and Elkner, 1990). Other authors (Aprea et al., 1997a) have shown that during treatment of vines with chlorpyrifos-methyl and re-entry of the vineyard, peaks of metabolite elimination mostly occurred within 16 h of the end of exposure.

Like other OP metabolites, urinary TCP may be used as an indicator of exposure to chlorpyrifos and chlorpyrifos-methyl, although available data is still insufficient to define biological exposure limits (Lauwerys and Hoet, 1993).

p-Nitro-phenol (PNP). PNP is a metabolic product of esterase cleavage of parathion, parathion-methyl and EPN (WHO, 1986b).

3-Methyl-4-nitro-phenol (MNP). MNP is a metabolite produced by esterase cleavage of fenitrothion (WHO, 1986b).

Malathion alpha-monocarboxylic acid (MCA) and malathion dicarboxylic acid (DCA). The mono- and dicarboxylic phosphoric acids derived from hydrolysis of diethylsuccinic ester in the lateral chain are the main urinary metabolites of malathion (Bradway and Shafik, 1977).

5.3. Carbamates

Carbamate pesticides have carbamic acid as their basic molecular structure. Addition of many types of radicals in the various reactive sites of this molecule has led to many products with herbicide, insecticide and fungicide properties. Each carbamate has its own chemistry and toxicology, though inhibition of cholinesterase activity is a biological effect typical of this group of pesticides.

5.3.1. *Blood cholinesterase activity*

Pre- and post-exposure levels of erythrocyte AChE is considered a good index of the effects of exposure to carbamates. Erythrocyte AChE is more sensitive than plasma ChE to exposure (WHO, 1986c). Inhibition of blood AChE in workers formulating carbofuran showed a significant correlation with airborne levels of pesticide when concentrations were above 0.1 mg/m^3 (Huang et al., 1989).

Cholinergic symptoms manifest in workers exposed to carbamates when blood AChE goes below 70% of pre-exposure levels (WHO, 1986c). Since recovery of activity is much faster than after exposure to OPs (carbamylation of the enzyme is readily reversed), blood samples should be obtained within 4 h of exposure and analysis should be done immediately (Coye et al., 1986b, WHO, 1986c).

5.3.2. *Unchanged compounds*

Measurement of unmodified carbamate insecticides in blood and/or urine has often been performed to confirm exposure in acute poisoning cases (Duck and Woolias, 1985; Lee et al., 1999; Burgess et al., 1994; Driskell et al., 1991). In fatal cases, unmodified compounds may be measured in various organs (Duck and Woolias, 1985).

5.3.3. *Metabolites*

Benomyl metabolites. The main benomyl metabolites are carbendazim (methyl 2-benzimidazole carbamate) (II), and methyl 5-hydroxy-2-benzimidazolecarbamate (III), which have been detected in experimental animals but never in biological fluids of exposed workers or the general population (Liesivuori and Jääskeläinen, 1984).

1-Naphtol (1NAP) and carbaryl. 1NAP is the main metabolite of carbaryl in humans, accounting for more than 85% of its metabolites in urine (WHO, 1994). 1NAP is also a metabolite of naphthalene and napropamide. It has been studied in exposed workers (Comer et al., 1975) and in the general population (Kutz et al., 1992; Hill et al., 1995).

2-Isopropoxyphenol (IPP). About 83% of propoxur absorbed is metabolised to IPP (Feldman and Maibach, 1974a; Feldman and Maibach, 1974b) which is excreted quite rapidly. After oral administration of propoxur in volunteers, 24.7% of the total dose is excreted in urine within 8–10 h (Dawson et al., 1964). After intraperitoneal administration in rats, 75% of the dose was recovered in urine as IPP (probably conjugated with glucuronic acid) within 24 h.

Propoxur is rapidly absorbed, metabolised and eliminated from the body. In volunteers tested with transcutaneous application of radioactive propoxur, radioactivity could be detected in urine within 4 h of application, reaching a maximum after 8–12 h and dropping to low levels within 48 h where it remained for more than 96 h. Dermal absorption was estimated at 15.9% (Feldman and Maibach, 1974a; Feldman and Maibach, 1974b). In a further study with volunteers (Machemer et al., 1982), quantities of IPP between 2 and 4 mg were excreted in the 24 h following the start of respiratory exposure to 3 mg/m^3 of propoxur lasting 4 h. The authors estimated a lung retention of about 40% (Machemer et al., 1982).

Urinary excretion of IPP has been documented in workers exposed to propoxur (Brouwer et al., 1993) and in the general population (Hill et al., 1995). Urinary IPP was monitored for 48 h after exposure of workers picking carnations in greenhouses in Holland (Brouwer et al., 1993). Total dermal and respiratory exposure showed a good correlation with the quantity of IPP excreted in 24 h when analysed by a multiple regression model, but respiratory exposure itself was not significant. Respiratory exposure contributed less than 20% to the total concentration of IPP excreted whereas skin exposure contributed about 80% (Brouwer et al., 1993).

In the absence of data on the relation between urinary levels of metabolites and effects of exposure, no health based biological limit can be proposed for this metabolite (WHO, 1982b).

Other metabolites. Carbofuranphenol (CFP, 2,3-dihydro-2,2-dimethyl-7-hydroxy-benzofuran) is a metabolite of several pesticides (e.g. carbofuran, benfuracarb, carbosulfan and furathiocarb).

Urinary excretion of 3-hydroxy-carbofuran has been found to correlate better than another metabolite, 3-ketocarbofuran, with exposure to carbofuran (Huang et al., 1989).

High urinary concentrations of metabolite I (2-dimethylamino-4-hydroxy-5,6-dimethylpyrimidine) and II (2-methylamino-4-hydroxy-5,6-dimethylpirimidine), excreted rapidly, have been detected in subjects exposed to pirimicarb (Verberk et al., 1990).

5.4. Pyrethroids (PYRs)

The term 'pyrethroids' is used for substances of natural origin, extracted from the pyrethrum plant, as well as various synthetic products with chemical structure similar to natural pyrethrins. It is therefore a large and varied group of products, the main characteristic of which is effectiveness as insecticides.

Pyrethroid insecticides may be absorbed by respiratory, cutaneous and digestive routes. An estimate of cutaneous absorption in humans *in vivo* suggests that generally less than 5% of the dose applied is absorbed (Wollen et al., 1992). Studies with experimental animals show that after exposure, pyrethroids are distributed throughout the body but just as quickly and completely excreted (IARC, 1991a; IARC, 1991b; IARC, 1991c; WHO, 1992). However the small amount that penetrates certain tissues, such as fat and the brain, may persist for several days after exposure (ElSalam et al., 1982).

5.4.1. *Unchanged compounds*

Occupational exposure to PYRs may be assessed by measuring intact compounds or their metabolites in urine. Because of their rapid metabolisation, determination in blood is only appropriate for recent high exposure. In a recent study (Leng et al., 1997), cypermethrin, cyfluthrin and permethrin were determined in plasma samples of 30 pest control operators. Pyrethroid concentrations were <5 µg/l (LOD) in all cases.

Other authors (He et al., 1988; Zhang et al., 1991) showed that in workers distributing fenvalerate and deltamethrin on cotton plants for a day, urinary deltamethrin could not be detected 12 h after the start of exposure, whereas fenvalerate could be determined up to 24 h after the end of exposure. Both compounds were detectable two days after the end of a period of exposure lasting 3 days. Urinary excretion of metabolites was greater than levels of unmodified deltamethrin, suggesting that the metabolites are better biological indicators of exposure than the unchanged compound.

5.4.2. *Metabolites*

3-phenoxybenzoic acid (3-PBA), 3-(4-hydroxy)-phenoxybenzoic acid (4OH-3PBA), 3-(2,2-dichlorovinyl)-2.2-dimethyl cyclopropane acid (DCVA), 3-(2.2-dibromovinyl)-2.2-dimethyl cyclopropane acid (DBVA), 2-(4-chlorophenyl)-3-methyl-1 butanoic acid (CPBA), 4-fluoro-3-phenoxybenzoic acid (F-PBA). Hydrolysis of the ester bond of permethrin, cypermethrin, deltamethrin, cyfluthrin and fenvalerate, produces acid metabolites and 3-phenoxy benzyl (4-fluoro-3-phenoxybenzyl for cyfluthrin) alcohol. The acid metabolites are: cis-trans-DCVA (permethrin, cypermethrin and cyfluthrin), cis-trans-DBVA (deltamethrin) and CPBA (fenvalerate).

Phenoxybenzoic compounds (3-PBA and 4OH-3PBA), derived from the alcohol group, are metabolites of permethrin, cypermethrin, deltamethrin and fenvalerate (He et al., 1988; He et al., 1991; Zhang et al., 1991; Chen et al., 1991); F-PBA is a metabolite of cyfluthrin (Leng et al., 1997).

A recent study with volunteers showed that after oral and dermal administration of cypermethrin, the four metabolites (cis- and trans-DCVA, 3PBA and 4OH-PBA) could be detected in urine for 5 days. The half-life of elimination was about the same for all four, but varied in relation to the route of administration (11–27 h for oral and 8–22 h for dermal). Differences in the proportions of the single metabolites were also noted: after oral administration, the total quantity of DCVA excreted was equivalent to that of total phenoxy derivatives. After dermal administration, however, DCVA was a quarter of total phenoxy derivatives (Eadsforth et al., 1988; Wollen et al., 1992). A different pattern or urinary excretion of metabolites of cypermethrin was also found in relation to route of administration for cis- and trans-DCVA: the ratio between the two forms was 1:1 and 2:1 after dermal and oral administration respectively (Wollen, 1993). These findings have aided interpretation of biological monitoring data and provided indications on the prevalent route of absorption during occupational exposure.

In a study (Chester et al., 1987) of subjects exposed to cypermethrin during aerial spraying of cotton, cis-DCVA was not detected in any urine samples whereas trans-DCVA was only found in some samples from workers who had been engaged in mixing. On the other hand, 3-PBA and 4-OH-PBA were detectable in all urine samples, the latter in higher concentrations. According to the authors, phenoxy-benzoic metabolites give the best indication of biotransformation of cypermethrin (Chester et al., 1987).

Cis-DCVA, trans-DCVA and 3-PBA were determined during distribution of permethrin (Llewellyn et al., 1996). All samples showed an increase in the concentration of metabolites after exposure, but 3-PBA was eliminated faster than the others.

3-PBA was detected in urine of a farmer exposed to fenvalerate while mixing the pesticide. Excretion decreased but remained detectable until the fourth day after the end of exposure (Aprea et al., 1997b). Since significant correlations have not been found between urinary excretion of metabolites and clinical effects (facial sensation and increased nerve excitability), these compounds can only be used as indicators of exposure (He et al., 1988; Zhang et al., 1991). Biological limits are not available for pyrethroid insecticides.

5.5. Organochlorine compounds (OC)

OC are a broad class of pesticides that were widely used as insecticides in the 1950s and 1960s. Their use was subsequently discontinued in many countries due to persistent contamination of the environment. They can be divided into three groups: benzene hexachloride isomers (e.g. lindane), cyclodienes (aldrin, dieldrin, endrin, chlordane, heptachlor, endosulfan) and DDT and analogues (methoxychlor, dicofol, chlorobenzylate).

Biological monitoring of OC exposure can be carried out by determination of intact compounds or their metabolites in blood and urine. Because of their persistence in the environment, most OC pesticides are ubiquitous pollutants and can usually be detected in biological samples from the general population.

5.5.1. *Unchanged compounds and metabolites*

After absorption, aldrin is rapidly converted to dieldrin. Exposure to both compounds has been assessed by measuring dieldrin concentrations in blood, serum, fatty tissue and milk. In certain studies it has been shown that adverse effects of aldrin and dieldrin were correlated with dieldrin concentrations in blood. The blood concentration of dieldrin below which adverse effects are not observed has been determined at 105 mg/l (WHO, 1989).

Technical chlordane is a mixture of α- and γ-chlordane, nonachlor and heptachlor. Biological monitoring of human exposure has been based on measurement of concentrations of chlordane and related compounds (oxychlordane, trans-nonachlor, heptachlor-epoxide) in blood, fatty tissue and milk. Concentrations of chlordane in blood have been correlated with the quantity of pesticide distributed during the period of biological monitoring and with the number of days the product was used (Saito et al., 1986).

Endrin is rapidly metabolised to 12-hydroxyendrin, and excreted as sulphate and glucuronide conjugate. Intact pesticide is usually undetectable in blood, fatty tissue and milk of occupationally exposed workers and the general population.

Exposure to heptachlor has been monitored by measuring its main metabolite, heptachlor epoxide, in blood, fatty tissue and milk of exposed subjects and the general population.

Technical grade hexachlorocyclohexane (HCH) consists of 65–70% α-HCH, 7–10% β-HCH, 14–15% γ-HCH and about 10% of other isomers and compounds. Lindane contains >90% γ-HCH. Exposure to lindane and HCH isomers has been monitored through concentrations of intact compounds in blood, fatty tissue and milk. Plasma levels of r-HCH have been used for biological monitoring of exposure to lindane. A biological limit of 20 mg/l lindane in blood has been recommended (WHO, 1982b).

Concentrations of DDT and HCH isomers in blood have been used to estimate dose after prolonged constant exposure. Levels of these compounds in cerumen have also been proposed as biological indicators of exposure. Although cerumen sampling is relatively easy, the data obtained only reflects cumulative exposure over a certain period of time, which may be months or years, but does not give any indication about recent exposure (Coye et al., 1986a). After absorption, DDT is largely transformed to DDE, and several intermediate metabolites have been measured in body tissues.

Chlorobenzylate and dicofol. The determination of unchanged chlorobenzylate and its metabolites (4,4′-dichlorobenzylic acid and 4,4′-dichlorobenzyldrol) requires oxidation to p,p′-dichlorobenzophenone. Determination of 4,4′-dichlorobenzylic acid in urine has been carried out in workers picking oranges treated with chlorobenzylate (Stamper et al., 1986). Urinary levels of 4,4′-dichlorobynzylic acid showed a significant correlation with residues of active ingredient on leaves, fruits and soil, sampled the day before biological monitoring. This result can probably be explained by the fact that absorption occurs prevalently by the dermal route, which

means that it takes several hours for the metabolite to appear in urine. The half-life of chlorobenzylate estimated in the study was about 2 days (Stamper et al., 1986).

5.6. Dithiocarbamate pesticides (DTC)

Dithiocarbamates are derived from carbamic acid by substitution of oxygens with sulphur. Again, substitution of various types of radical has differentiated many products, imparting specific physicochemical and toxicological properties. These products are particularly effective fungicides and do not significantly inhibit cholinesterase activity.

These substances can be divided into thiurams (thiram, methiram, disulfiram), dimethyldithiocarbamates (ferbam, ziram) and alkylenbisdithiocarbamates (ethylene and propylene). The metabolic pathway of DTC is very complex, producing a great number of metabolites. One is carbon disulfide, which is further partially metabolised to 2-thiazolidinethione-4-carboxylic acid (WHO, 1988). These compounds have both been determined in urine of occupationally exposed and unexposed subjects, though they are not specific indicators of exposure to DTC. Ethylenethiourea, on the contrary, is the specific metabolite of ethylenebisdithio-carbamates (EBDC) (mancozeb, zineb, maneb, etc.) and is the most promising indicator of exposure for biological monitoring.

5.6.1. *Metabolites*

2-Thiazolidinethione-4-carboxylic acid (TTCA). CS_2 is metabolised to TTCA by addition to the cysteinyl-SH group of glutathione and subsequent ring condensation (Bus, 1985). Consumption of brassica vegetables (Simon et al., 1994) is a non negligible source of urinary TTCA. The pesticide captan may also produce TTCA during metabolisation (van Welie et al., 1991). TTCA is a well known marker for biological monitoring of exposure to CS_2 (BEI 5 mg/g creat.) (ACGIH, 2002), and the WHO has also suggested this indicator for monitoring exposure to DTC (WHO, 1996).

Ethylenethiourea (imidazolidin-2-thione, ETU). Besides being an environmental, animal and human metabolite of EBDCs, ETU is an impurity of EBDC formulations (Bontoyan et al., 1972; Jordan and Neal, 1979). It is also used in vulcanisation of rubber. It has long-term effects characterised mainly by antithyroid activity (WHO, 1988). NIOSH classifies ETU as carcinogenic for humans; OSHA classifies it as suspected carcinogen, as does IARC (IARC, 1974; IARC, 1983).

Cutaneous and digestive absorption of EBDCs is generally relatively low (WHO, 1988). After elimination of the metal and transformation into ethylenethiuram disulphide, the substance is converted to monosulphide and ethylenebisisothiocyanate and/or ETU. CS_2 may be formed in various phases of the metabolic breakdown of EBDCs. ETU is the main urinary and fecal metabolite detected in experimental animals after oral administration of EBDC (Camoni et al., 1984). Rats fed a single oral dose of zineb (50 mg/kg b.w.) began to excrete ETU in urine 6 h later, with

a peak at 24 h when excretion was about 52% of the total amount of ETU excreted in urine. Low levels of ETU were measured in urine up to 15 days after administration, however only 5.1% of zineb is eliminated in urine as ETU. Fecal levels of ETU are no longer detectable 72 h after administration. Quantities of ETU eliminated with the feces and urine in the first 48 h were 14% and 86% respectively (Camoni et al., 1984). When mice were given 14C-maneb by gastric intubation, the proportion of the dose recovered in urine and feces within 48 h was 91.0–92.7% and 7.3–9.0% at different dose levels. In the two studies, ETU constituted 15.8% and 7.8% of the part excreted in urine and the rest was more polar metabolites (Jordan and Neal, 1979).

ETU metabolism also seems characterised by rapid biotransformation in mammals. The peak of elimination of metabolites in expired air was observed 5 h after oral administration to rats, and 80–95% of the dose was eliminated in the urine within 24 h of administration (Kato, 1976).

Various authors have determined urinary ETU in subjects exposed to mancozeb and zineb (Aprea et al., 1998; Kurttio and Savolainen, 1990; Kurttio et al., 1990; Sciarra et al., 1994; Colosio et al., 2002) and in groups of the general population (Aprea et al., 1996b; Aprea et al., 1997c).

Workers treating potatoes with EBDC excreted ETU slowly with concentrations in the range 0.09–2.5 mg/mmol creat the first day after treatment and <0.01–0.2 mg/mmol creat 22 days after treatment. The elimination half-life, calculated on a graphic basis, was about 100 h and was probably related to exposure time (0.5–7 h) and slow dermal absorption of ETU and EBDC during and after the work shift. The wide variations in urinary concentrations depended on the personal protection used, time engaged in treatment and personal hygiene habits of the workers (Kurttio and Savolainen, 1990; Kurttio et al., 1990).

Subjects exposed to mancozeb during industrial formulation showed urinary excretions of ETU in the ranges 7.8–644.4 mg/g creat and 14.2–104.4 mg/g creat in two plants where commercial products containing 80% and 45% mancozeb, respectively, were formulated. Urinary ETU showed a significant correlation (multiple regression model) with respiratory and cutaneous doses of ETU and mancozeb (Aprea et al., 1998).

It can be concluded that ETU is a very sensitive indicator for evaluating exposure to EBDCs, but urine samples must be obtained for at least 24 h after the end of exposure. Since ETU is detectable in urine of the general population results of biological monitoring should be compared with those of adequate reference groups or baseline levels of the workers monitored. Biological exposure limits for ETU are not available.

5.7. Herbicides

Herbicides have a wide range of chemical structures, including organophosphates, carbamates, thiocarbamates, dithiocarbamates, triazine and many others.

5.7.1. *Unchanged compounds and metabolites*

2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chloro-phenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), pichloram, mecoprop, dichlorprop. 2,4-D and MCPA may be absorbed by respiratory, cutaneous and digestive routes. They are distributed in the body but there is no evidence of accumulation in organs and tissues (WHO, 1984a; IARC, 1986). These compounds are scarcely metabolised and are excreted largely unchanged in urine.

After administration of a single oral dose to volunteers, about 80% of 2,4-D was excreted unchanged in urine and the rest in conjugated acid-labile form (IARC, 1986). Other experimental data (Donovan et al., 1984) shows that humans begin to eliminate 2,4-D in urine as soon as 2 h after oral administration. Maximum urinary excretion occurs on the first and second days after ingestion. After cutaneous application (Feldman and Maibach, 1974a; Feldman and Maibach, 1974b), maximum urinary excretion occurs on the second and third days. More than 90% of the absorbed dose of 2,4-D is excreted in urine within 5 days, and the speed of elimination probably depends on urine pH (Lavy and Mattice, 1986). Slow excretion of the compound in urine, with a half-life in the range 12–22 h, has also been observed during distribution of the compound (Aprea et al., 1995; Knopp and Glass, 1991).

Similar studies for MCPA (Fjellstad and Wannag, 1977; Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b) showed faster excretion than for 2,4-D: 40% of the dose was excreted in urine in the first 24 h and 80% in the first 5 days after a single oral dose. After skin application of MCPA, a slow increase in plasma concentrations was observed with a maximum after 24 h. In urine, slow excretion that continued for more than 5 days was observed, with a peak 24–48 h after application. The biological half-life was 12–72 h (Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b).

Urinary 2,4-D and MCPA are very sensitive indicators of exposure of these two compounds. If urine collection continues for 24 h from the end of exposure or if spot urine samples are obtained the morning after exposure, the two compounds can be used as indicators of dose. The pattern of urinary levels of MCPA, dichlorprop, mecoprop and 2,4-D during exposure in agriculture have been studied by various authors (Kolmodin-Hedman et al., 1983a; Kolmodin-Hedman et al., 1983b).

Glyphosate. Exposure to glyphosate may be monitored through unchanged compound and aminomethyl-phosphonic acid in urine (Lavy et al., 1993; Jauhiainen et al., 1991). Studies in monkeys showed that 89% of the dose absorbed through the skin is excreted in urine within 5 days (Wollen, 1993). To evaluate exposure to glyphosate in conifer nurseries, the unchanged substance has been assayed in urine (Lavy et al., 1992) but the compound was never found in the samples analysed.

2,6-Diethylaniline (DEA) and 2-(1-hydroxyethyl)-6-ethylanyline (HEEA). Urinary DEA and HEEA are metabolic products of alachlor, after alkaline hydrolysis. Studies in monkeys have shown their relative proportions to be 8:2 and a similar relation-

ship is reported in urine of exposed human subjects (Wollen, 1993). This confirms that primates can give reliable results for the estimate of absorbed dose in exposed subjects, in the absence of studies on volunteers.

Diquat and Paraquat. Determination of the unchanged quaternary ammonium compounds in blood and urine has been used to monitor human exposure (WHO, 1984b). Urinary concentrations of paraquat in exposed subjects were often less than 0.01 mg/l, though concentrations above 0.73 mg/l were found after incorrect use in tropical agriculture. Urinary levels decreased rapidly during the first 24 h after exposure, remaining detectable for several weeks (WHO, 1984b).

Fluazifop. Field studies after use of enantiomer R of fluazifop-butyl showed urinary levels of fluazifop in the range 2.7–22 mg/day in exposed subjects (Wollen, 1993). This data is in line with previous studies on volunteers, in which 90% of an oral dose of this compound was eliminated in urine as the acid metabolite fluazifop. Other studies of dermal administration of commercial formulae showed an inverse relation between dose and quantity absorbed (Wollen, 1993).

5.8. Other compounds

5.8.1. *Unchanged compounds and metabolites*

Tetrahydrophthalimide (THPI). In mammals captan is primarily metabolised to thiophosgene and tetrahydrophthalimide (THPI) which are excreted in urine (van Welie et al., 1991). Thiophosgene is conjugated with glutathione (GSH) and excreted as 2-thiazolidinethione-4-carboxylic acid (TTCA) after enzyme degradation and ring closure. Studies with rats dosed orally with 60 mg captan have shown that 12.7% was excreted as THPI in 24 h (Wollen, 1993). In the human body, THPI is a minor metabolite of captan since only 2.2% of an oral dose of 0.1 mg/kg was eliminated in urine in 24 h (Wollen, 1993).

Urinary THPI was determined in subjects exposed to captan while harvesting fruit (de Cock et al., 1995; de Cock et al., 1998, Krieger and Dinoff, 2000). The dose absorbed was evaluated through excretion of THPI in 24 h.

THPI may be used as a quite sensitive indicator of exposure to captan but not enough data is available for its use as a biological indicator of dose.

4-chloro-o-toluidine (CT). Chlordimeform is an acaricide-insecticide. Occupational exposure has been assessed by measurement of the unchanged compound and 4-chloro-o-toluidine in urine (that account for 70–90% of chlordimeform excretion products). These two compounds increase rapidly in urine of spray operators with a peak 4–6 h after exposure (Wang et al., 1987). The amount excreted gradually increases during three days of exposure and begins to decrease immediately after the end of exposure, going back to the pre-exposure level within 5 days. Total urinary excretion of both compounds is correlated with dermal exposure and can be used as biological indicator of exposure (Wang et al., 1987).

Pentachlorophenol (PCP). PCP concentrations in blood and urine have been proposed as indices to monitor occupational exposure. Biological Exposure Indices (BEI) for PCP have been recommended by ACGIH (ACGIH, 2002) and Biological Tolerance Values by the DFG (DFG, 1993). PCP only occurs in urine as a consequence of exposure to the compound. The adsorbed dose is excreted largely unmodified (86%): 74% free, 12% conjugated with glucuronic acid (Williams, 1982; WHO, 1987). The timing of urine sampling was not found to be critical and a single spot sample was sufficient in most biological monitoring programmes (Coye at al., 1986a).

Dinitro-o-cresol (DNOC). To monitor exposure to DNOC, the substance itself was determined in blood (Coye at al., 1986a). Agricultural use of this pesticide has been curbed due to its high toxicity for humans and plants. As a consequence of slow excretion, blood levels of DNOC increase after repeated exposure and are correlated with adverse effects (Coye at al., 1986a). Determination of DNOC in blood is widely used to evaluate exposure and clinical course in cases of poisoning. Blood concentrations of 10–20 mg/l DNOC are regarded as alarm levels (WHO, 1982b).

Chlorotriazine. The most representative compound in this group is atrazine. Since atrazine metabolism gives rise to bidealkylated (80%), deisopropylated (10%) and deethylated metabolites, intact compound and metabolites can be detected in body fluids of exposed subjects (Catenacci et al., 1990; Catenacci et al., 1993). In urine samples of sprayers, the mercapturic acid conjugate of atrazine was found to be the major urinary metabolite, having concentrations at least 10 times higher than those of dealkylated products and the parent compound.

Since other chlorotriazines (simazine, propazine, terbutylazine) follow the same metabolic pathway as atrazine, urinary excretion of bidealkylated, deisopropylated and deethylated metabolites are not compound-specific. The unmodified compound in urine represents only a minor portion of the absorbed dose, however its determination may be useful for qualitative confirmation of exposure.

5.9. Interpretation of the results of biological monitoring

The milestone of the interpretation of biological monitoring data is a good knowledge on dose response and dose effect relationships. For most active ingredients this knowledge is lacking, and biological indices of exposure are available only for few compounds (see Table 12).

Under these conditions, it is difficult to evaluate potential health risk. However, biological monitoring may be useful to assess absorbed dose through comparison with pre-exposure levels or reference values obtained for the general population (biological reference values). Since these values are the result of background environmental exposure, in preventive strategies they are target values to control the additional risk caused by occupational exposure. In studies aimed at assessing the efficacy of personal protection, comparison with reference or pre-exposure values makes it possible to evaluate whether absorption is continuing (Aprea et al., 1994a;

Table 12. Recommended biological limit values.

Compound	Biological indicator	BEIs[a]	BATs[b]	HBBLs[c]
Acetylcholinesterase inhibiting pesticides	AChE	70% of individual's baseline	70% of the reference value	70% of the reference value
DNOC (dinitro o-cresol)	DNOC in blood	–	–	20 mg/l
Lindane (HCH)	HCH in blood	–	0.02	0.02 mg/l
	HCH in plasma/serum	–	0.025	–
Parathion	p-Nitrophenol in urine	0.5 mg/g creat	0.5 mg/l	–
	AChE	70% of individual's baseline	70% of the reference value	–
Pentachlorophenol (PCP)	PCP total in urine	2 mg/g creat	–	–
	PCP free in plasma	5 mg/l	–	–
Arsenic elemental and soluble inorganic compounds	Inorganic arsenic plus methylated metabolites in urine	35 µg As/l		

[a] BEI: Biological Exposure Index (ACGIH, 2002); [b] BAT: Biological Tolerance Value (DFG, 1993);
[c] HBBL: Health-Based Biological Limit (WHO, 1982b).

Aprea et al., 1994b; Aprea et al., 1995; Aprea et al., 1997a; Aprea et al., 1998; Aprea et al., 1999b, Aprea et al., 2001a). This information is particularly useful for exposure to active ingredients with known or suspected long-term toxicity.

6. INDIVIDUAL PROTECTION OF WORKERS

Measures to reduce exposure and occupational risk depend on the work task performed, weather conditions and money available to purchase protective clothing or devices. For a given exposure potential, use of personal protection (PP) can considerably reduce real exposure, though worker hygiene, cleanliness and maintenance of protective equipment are important. A bad habit is to return home with work clothes because it protracts the period of skin contact with pesticide residues on clothes, favouring absorption and contamination of other materials.

Besides the choice of appropriate PP, the operations required for its decontamination before reutilization are important. Sometimes thorough washing is not sufficient to remove pesticide residues, which after use tend to pass through the fabric more readily (reduced breakthrough time).

To prevent skin contamination in workers using pesticides, the first barrier may be a closed tractor cabin with filtered and conditioned air, or impermeable overalls.

The second barrier may be work clothes (cotton overalls, trousers, t-shirts or shirts with long or short sleeves, shoes, hats, gloves, etc.).

Common reasons why total-cover garments may not be completely impermeable to toxic substances include construction defects, and discontinuities such as openings and zips. Reasons why closed cabins may not provide good protection may be residues inside the cabin, or entry through seals and gaskets rather than the air filtration system.

For workers re-entering treated areas, use of PP is more complex. These operations are often done in the field in summer, when high temperatures make it impracticable to wear much protective clothing. To obviate this, some authors have proposed use of a light cotton tunic, buttoned on the shoulders and reaching the knees (Aprea et al., 1994b).

For workers exposed to pesticides in industry (synthesis, formulation and packaging), use of impermeable clothing on top of normal clothes is generally limited to times of maximum exposure, for example when manual intervention is called for on the plant. Operations of control of closed cycle automatic plants are usually carried out with common work clothes (Aprea et al., 1998).

Clothes to protect skin must be of the right size. Gloves too large may reduce agility of movement and favour entry of pesticide inside the glove. Gloves which are too small may compress the hands or tear. Whether long or short gloves are worn depends on the type of exposure. Important variables are glove thickness and material. In some products, the palm of the hand is treated differently from the rest of the glove to provide greater protection to certain skin areas. In certain cases, the use of gloves may give rise to problems such as excess sweating, reddening of the skin and occlusion phenomena. The causes of these reactions may sometimes be additives in the glove itself. Some workers use medicated powder inside gloves to absorb perspiration. This may create problems because compounds in the powder (e.g. eucalyptus oil) may favour irritation of the skin. A solution often used is to wear thin cotton gloves under the chemical glove to absorb perspiration (Aprea et al., 1994a; Aprea et al., 1998). A disadvantage is the need to change the cotton gloves frequently.

The use of protective shoes made of material similar to that of gloves is a relatively recent practice. Shoes are rarely decontaminated and are the articles most frequently worn home. To avoid the spread of contamination, overshoes could be worn on the job.

To limit respiratory exposure, the first protective barrier may be closed cabins or closed helmets or masks with combined filters for dusts and vapours. Helmets not only ensure respiratory protection but also complete cover of the skin of the head, preventing pesticide aerosol from entering the mouth and nose where they would be swallowed and absorbed by the digestive system.

For workers re-entering sprayed areas, devices for respiratory protection are rarely provided (Aprea et al., 1994a, Aprea et al., 1998). In some situations felt masks that stop particulate released on handling of fruit, flowers or leaves containing pesticide residues has been associated with a reduction in urinary excretion of metabolites (Aprea et al., 1994b).

For workers exposed to pesticides in industry, use of respiratory protection is gen-

erally limited to times of maximum exposure, for example for manual mainte-
nance of the plant. Operations of control of closed cycle automatic plants are usually
carried out with common work clothes (Aprea et al., 1998).

6.1. Personal protection (PP): choice and testing of efficacy

Among the wide range of products available commercially, choice of the material
of which PP devices are made must be done in a critical and informed manner
(NIOSH, 1990). Information required includes:

– Detailed composition of the mixture used in the field. Other ingredients may
  interact with skin protective devices favouring passage of pesticide. For respi-
  ratory protection, substances other than the pesticide may saturate filters and
  adsorbent materials, reducing protection against the toxic substance.
– Physical state and chemical properties of the substances used. If a substance is
  present in the vapour phase, devices that protect the whole skin surface must
  be used.
– Work task and manner of exposure. It is necessary to know the body areas
  which may come into contact with the pesticide and whether operations are
  performed which could damage PP. If contact with the substance is occasional,
  PP could be removed or replaced immediately after exposure. In the case of
  frequent or continuous contact, PP must ensure protection for the whole work
  shift. For tasks requiring manual dexterity, PP may be a nuisance and clothing
  that covers the whole body may cause thermal stress.
– Re-utilisation. If PP must be removed and re-utilised, appropriate decontamina-
  tion procedures are necessary.
– Ambient conditions. These factors are important for thermal stress and changes
  in permeation and breakthrough which may occur with temperature.

The efficacy of PP may be tested in the laboratory or in the field.

Laboratory tests of PP usually measure breakthrough time, breakdown of material
and degree of penetration of liquids. To evaluate breakthrough, a two chamber
test is used. The material to be tested acts as a barrier between the two. The chemical
substance is placed in chamber in contact with the external surface of the material
and permeation of the substance is measured in the other. This method tests con-
tinuous contact with the substance, measuring worst conditions which go with fastest
breakthrough.

To evaluate breakdown of material, a screening test is used (weight change, visual
examination) after exposure. Materials that show a large weight increase, decolora-
tion, deterioration and so forth are not regarded as suitable.

There is no consensus on the manner of conducting laboratory tests for PP since
in most cases, tests do not reflect operating conditions. For example, PP generally
cause an increase in skin temperature and sweating which breakthrough tests do
not take into consideration. Temperature and sweating should be simulated in these
tests. Moreover, since lab tests are done with pure substance, they do not take the
presence of other ingredients, which may be more aggressive for clothing, into
account. These ingredients may damage the fabric of the clothing, allowing the toxic

ingredient to pass through. To obviate the problem of mixtures for which all components have not been tested, it is possible to reduce the utilization time of garments. For example, it may be appropriate to replace gloves periodically during the work shift.

All PP contain some type of additives, such as plasticizers and fillers. Certain solvents in pesticide formulations may extract these additives. Laboratory tests should also address this possibility.

Other laboratory tests are concerned with filters used for respiratory devices and in cabins. These tests are usually done with substances other than pesticides, enabling the producers to guarantee working of the filter for a certain number of hours, provided they are used correctly.

Testing of PP in the field involves application of the methods used for evaluation of skin exposure (pads, hand washing, fluorescent tracers, biological monitoring). Field tests of respiratory protection devices may also use air monitoring, wipe tests and analysis of biological matrices.

6.2. Field testing of PP

6.2.1. *Closed cabins used during treatment of crops*

It has been reported that if correctly used, closed cabins intercept up to 90% of total potential exposure (Krieger et al., 1990). Few studies have evaluated the efficiency of filters for tractor cabin air inputs under real conditions of use. The lifetimes advised empirically by manufacturers are in the interval 200–400 h. In a recently published study (Aprea et al., 2001b), penetration of mancozeb into a cabin was investigated in the field in relation to age of input air filter. The filter was a Pan Clean AX7228: the result obtained in tractors with tracks showed a penetration of $5.6 \pm 10.0\%$ under 200 h and $48.7 \pm 27.0\%$ when the filter was used beyond 200 h. For tractors with wheels, data is only available for less than 200 h of life, with a mean penetration of $1.4 \pm 2.1\%$. The low protection offered by filters used for more than 200 h is probably due to high environmental dust levels, especially for tractors with tracks. Deposition of particulate on the filter material can cause unfiltered air to enter by routes other than through the filter, reducing filter efficiency.

6.2.2. *Protective clothing*

Various studies have been conducted to determine the barrier efficacy of various protective garments. In a study by Davis et al. (Davis et al., 1982), the protection offered by cotton overalls was compared with that of ordinary protective clothing during preparation and application of ethion to citrus trees. Pads of alpha-cellulose were worn inside and outside the protective clothing. On the average, the overalls reduced skin exposure by a factor of seven for mixing and a factor of 20 for spraying. In a later study (Nigg et al., 1986) the protection afforded by Tyvek overalls was compared with that of common clothes (long sleeve shirt and cotton or polyester trousers) during mixing and spraying of dicofol on citrus trees (alpha-cellulose pads were worn under and on top of the overalls). Skin exposure, excluding

hands, was reduced to 38% during spraying and 40% during mixing. A further study (Keeble et al., 1987) for mixing and spraying of azinphos-methyl, showed that Gore-Tex overalls reduced contamination to 13%, whereas overalls in Tyvek covered with Saranex were associated with 21% skin contamination with respect to the potential value (100%) recorded outside the overalls. Again pads inside and outside the protective clothing were used. Another study (Fenske, 1988) using fluorescent tracers during spraying of pesticides showed that cotton or Tyvek overalls treated with olefin reduced skin exposure to 50% and 25% respectively, compared to cotton or polyester t-shirts.

Various other studies have been conducted with the aim of evaluating contamination of various areas of skin, covered and uncovered by certain protective clothing. In a study (Aprea et al., 1994b) conducted during fixing of ornamental plants treated with fenitrothion in greenhouses, pads of filter paper applied directly to the skin were used to demonstrate contamination of uncovered skin (head and neck) in the range 7.7–65.2 nmol/day. Contamination of uncovered skin showed a significant correlation with concentrations of active ingredient in airborne particulate. Use of protective clothing consisting of cotton overalls, cotton apron and work shoes, worn over personal underwear (knickers, socks and cotton t-shirt) was associated with undetectable contamination of covered skin. This indicated that under those conditions, the protective clothing provided almost complete protection. Protection of the hands by means of cotton gloves worn over rubber gloves was similarly effective because the doses of pesticide found in hand wash liquid, only 0.8–17.4 nmol/day, were about half the doses found on the cotton gloves. Since skin protection was so effective, the respiratory dose, 62.7–494.3 nmol/day, was on average more than 80% of the actual total dose and more than 91% of the total absorbed dose. The total dose, which was in the range 1.0–8.8 nmol/kg b.w./day, was below the acceptable daily intake (ADI) of fenitrothion, considered to be 10.8 nmol/kg b.w./day.

A similar study with chlorthalonil (Aprea et al., 2002) showed respiratory doses in the range 20.47–76.34 µg/day and cutaneous doses in the interval 121.74–847.41 µg/day. Hands accounted for 52% of the latter, uncovered skin for 3% and skin covered by clothing for 45%. The parts of the body receiving the greatest contamination were the thighs and anterior hips, followed by arms and forearms.

In another study (Aprea et al., 2001a) in greenhouses during various manual operations such as positioning, spacing, selection and watering of ornamental plants treated with fenitrothion and/or omethoate and/or tolclofos-methyl, cotton overalls and work shoes worn over socks and t-shirt considerably reduced exposure of covered skin to omethoate, but protected relatively little against tolclofos-methyl. In this case, inhaled dose was 4.5%, 49.5% and 9.9% of the total dose for omethoate, fenitrothion and tolclofos-methyl respectively. For the first two, the main contribution to cutaneous dose was contamination of the hands which were protected by rubber gloves, accounting for 65% and 93% of the total cutaneous dose. For tolclofos-methyl, the hands contributed 33% and skin covered by clothes, 63%. The constant presence of this pesticide on covered skin is presumably due to penetration of clothes, which were only changed once a week.

In another study carried out in the open field during thinning of juvenile fruits

on peach trees previously treated with azinphos-methyl and chlorpyrifos-methyl (Aprea et al., 1994b), hand contamination was investigated in relation to the type of gloves worn. Since potential dose was the average of that found on the hands of a group of workers who did not wear gloves (2907.9 nmol/day), it can be said that cotton gloves reduce contamination to 22%, cotton gloves impermeabilised on the palms and backs to 29% and rubber gloves to 0.4%. Protection afforded by cotton gloves was similar to that of impermeabilised cotton gloves. In our opinion, the differences observed are due to variations between operators and assessment procedure.

Another very recent study (Creely and Cherrie, 2001) looked at the efficacy of three types of gloves during handling of permethrin, showing that PVC gloves had a protection factor of 96 compared to 200 or 470 for vinyl gloves of different thickness and length. Thickness (1.2 mm), length (270 cm) and poor flexibility made PVC gloves less effective. The more effective of the two vinyl glove types were those 0.44 mm thick and 330 mm long. Increased length to 370 mm did not make up for the tiny increase in thickness to 0.48 mm, the protection factor dropping by half. This demonstrated the importance of adherence of gloves to hands during activity. Gloves that did not adhere allowed pesticide to enter, causing significant hand contamination.

6.2.3. *Use of biological monitoring for integrated evaluation of efficacy of PP*

Compared to environmental monitoring, biological monitoring has the advantage of demonstrating absorption of pesticides by the body, summing all routes of penetration. Although this method does not characterize exposure qualitatively, it is the only possibility when we want to determine whether protection is effective or when we want to evaluate the effect of devices that cannot be studied in the field by other methods. For example, respiratory protection such as helmets and masks with single or combined dust-vapour filters are difficult to assess in the field because it is practically impossible to obtain air samples inside the mask or helmet.

In a study (Aprea et al., 1993) on workers treating vines with mancozeb, absorption of active ingredient and its degradation product were estimated by assay of ethylenethiourea in urine. During the first cycle of measurements, the workers were not required to wear any protection beyond what they normally wore (long pants and shirt or cotton overalls but no gloves). In the second and third cycles they wore helmet or mask with filters for dusts and vapours. In the third cycle they wore skin protection in the form of overalls of Tyvek or cotton, gloves and rubber boots. The results showed that respiratory protection reduced absorption by 97.4%, mean excretions dropping from 232.0 to 6.1 µg/l. Skin protection in the third cycle of measurements led to mean excretions of 5.8 µg/l.

A further study (Aprea et al., 1994b) was concerned with workers thinning juvenile peaches in an orchard treated with azinphos-methyl and chlorpyrifos-methyl. Absorption of active ingredients was evaluated by assay of urinary alkylphosphates in five groups of workers who wore different protective devices. Urinary excretion was compared with that of a control group selected in the area. All subjects

monitored wore normal clothes such as trousers, closed shoes and t-shirt. The first group did not wear any additional protection, whereas the others wore a knee-length cotton tunic with long sleeves, buttoned on the shoulders. Group 2 also wore cotton gloves, group 3 cotton gloves and felt mask, group 4 impermeabilised cotton gloves and felt mask, and group 5 rubber gloves and felt mask. In group 1, mean excretions exceeding 3500 nmol/g creat indicated urinary alkylphosphate levels about 25 times higher than in controls. In the other groups, mean levels of excretion were 2–4 times higher than in controls. Comparisons between groups 2 and 3 showed that the felt mask reduced absorption by about 60%. Comparisons between groups 3, 4 and 5 showed the efficacy of the different types of gloves. Group 3 had the lowest excretion levels which suggests that cotton gloves are probably the most comfortable.

7. CONCLUSIONS

The present review sets out the techniques and procedures available today for evaluating cutaneous and respiratory exposure to pesticides. The choice of monitoring strategy depends on the type of compound considered and working conditions, although a general orientation is to carry out environmental and biological monitoring simultaneously. Such studies can:

– provide information on the relation between estimated exposure doses and urinary excretion of metabolites;
– evaluate the contributions of cutaneous and/or respiratory exposure to total absorbed dose;
– check the appropriateness of biological indicators used at various exposure levels;
– provide further data when that provided by environmental or biological monitoring proves inadequate;
– provide data which can be used by data banks to build models.

The number of samples (environmental and biological) to obtain must be established on the basis of the necessary statistical confidence levels. The variability of exposure in the field can be evaluated more accurately increasing the number of subjects rather than repeating measurements more often in the same workers. However, in the case of biological monitoring, it is advisable to continue collecting urine in a given subject for a certain period of exposure, equal to about four times the half-life of the substance. This is useful for evaluating elimination kinetics and possibly absorbed doses.

A difficulty that may be encountered in field studies is the lack of standardized methods for estimating dermal and respiratory doses and for assaying pesticides and their metabolites in biological fluids. Although biological monitoring is hardly a routine procedure, in many exposure situations it may be the only possibility. There continue to be few established biological limit values and little knowledge of the toxicokinetics of the various pesticides in relation to dose and routes of absorption.

Experiences conducted by various researchers have revealed the extent to which

working conditions and individual protection devices differ in relation to situations. An undoubted advantage of the simultaneous availability of both environmental and biological monitoring data is to enable optimization of safety procedures for workers with a view to a progressive reduction of the risk of exposure. In this framework, the determination of reference values for various biological indicators is a useful interpretative auxiliary for estimating residual risk.

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CROP QUALITY UNDER ADVERSE CONDITIONS: IMPORTANCE OF DETERMINING THE NUTRITIONAL STATUS

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1. INTRODUCTION

During plant growth, environmental changes affect nutrient uptake and plant development. Moisture supply, temperature, light and soil properties influence element availability as well as nutrient uptake, concentration and accumulation in the plant. Different plant species vary not only in the rate at which they absorb an available nutrient, but also in the manner by which they spatially distribute the element to different organs in the same plant. However, not all the nutrients present in the soil or applied in a soil system are available to the plant. The importance of nutrient uptake to crop productivity is assessed not only from the standpoint of the accumulation of dry matter but also in terms of the economic return and the environmental pollution by nutrient leaching. Interaction between nutrients can occur on the root surface or within the plant, and in crop plants, such interactions are generally measured as growth response, either positive or negative. When nutrient combinations prompt a growth response greater than the sum of their individual effects, the interaction is positive and the nutrients are synergistic, whereas when the combined effect is less the interaction is negative and the nutrients are antagonistic (Fageria et al., 1997a).

Interactions among nutrients, both in the growth medium of the plant as well as within plants, can lead to nutritional imbalances and therefore to deficiency or toxicity in elements needed for good development, decreasing plant growth and crop yield (Kochian, 2000). Thus, the deficiency of an element, as well as toxicity not only affects plant growth, but also induces morphological changes that can resemble effects caused by pathogens. Besides the problems involving micronutrient deficiencies or toxicity there is another crucial factor affects the plant growth and development: the crop age. The physiological age of the plants, affects the crop performance by inducing alterations in the nutritional status in the different plant tissues and organs. Usually, as plant age progresses, the nutrient contents, on a dry-weight basis, decreases (Mozafar et al., 1993), due to the improved dry-weight production during growth (Jarrel and Beverly, 1981). Nutrient contents also depends on whether elements are mobile (N, K,) or immobile (Ca, Mg), since in older plants the foliar levels of N, P, K, . . . tend to fall, as long as the levels of Ca, Mg, etc. rise, although such trends are not uniform along the entire leaf section (Mills and Jones, 1996).

Besides these considerations, dependent mainly on external factors, nutrient uptake and plant growth also depend on genetic factors. In fact, differences in

nutrient uptake and use between plant crops and even between cultivars within the same species are well-established (Siddiqui et al., 1987). Thus, in recent years it has been demonstrated that the most important approach is to identify the genetic specificity of the plant mineral nutrition (Saric, 1987). Genetic variability in relation to yield is defined as the hereditary characteristics of species or cultivars that cause differences in yield despite favorable or unfavorable environmental conditions (Fageria et al., 1994b). These genetic factors inevitably determine the productivity of the species or cultivar, and thus, together with fertilizer application, determine crop yield. The quantitative evaluation of these factors and their interactions can help in developing systems to optimize the production. Therefore, we can alter not only the growing media and the cultivation practices but also the physiological characteristics of the plant to achieve an optimal yield in a given environment.

Since crop yield in experiments under controlled conditions is usually higher than under field conditions, we undertook a series of experiments designed to explore the influence of different crop conditions on different species and cultivars. Conducting controlled *versus* field experiments, using horticultural and economically important species that respond differently under adverse conditions, we tested variations in fertilizer application, fungicides, bioregulators or growth-regulator substances, different environmental conditions, genetic variability, and grafting processes. We studied the influence of all of these parameters as factors affecting the plant growth and development. For each situation, we here conduct a nutritional diagnosis study as well as analysis of response of yield and quality parameters of the crops.

2. GROWTH CONDITIONS AFFECTING THE NUTRITIONAL STATUS, AND THE FINAL YIELD AND QUALITY OF CROPS

To ascertain the nutritional status of a plant or a crop, we need to know the appropriate conditions for optimum growth and development – that is, maximum yield and quality in agricultural plants with certain economic impact. Since environmental conditions vary among countries, regions and even in the same cultivation area, and in different seasons, field experiments on plant nutrition are not always applicable to different geographical areas. Thus, crop experimentation at different sites provides the researcher a wide range of fully controlled environmental factors (i.e., growth-chamber *vs*. greenhouse or the open field). In this way, we can establish the limits of growth, development and yield of a crop, thereby gaining a clearer idea of the nutritional status in plants of economic interest under specific conditions.

2.1. Experiences under controlled conditions

2.1.1. *Growth-chamber experiments*

The use of growth-chambers allows the control and maintenance of certain environmental conditions, and thus enables the quantification reaction to a determined

stress, biotic or abiotic, without existing external interferences. Important factors affecting the plant growth and development are the interaction between nutrients and the pesticide application, as we cited above. The effect of both factors could be also different depending on the environmental parameters in the study or growth zone. We designed and studied the influence of both examples in growth-chamber or environmental-chamber experiments, to analyze the nutritional relationship between phosphorus and calcium supplies, as well as the effect of the application of one pesticide, *Carbendazime*, on tobacco crops.

P-Ca interaction

When we need to ascertain the specific effect of the application of one nutrient to know the appropriate levels to use later in the commercial activity, and the levels inducing toxicity or deficiency in a given crop, and also to study the interaction between elements, the growth-chamber is the ideal method to test and verify all these issues.

We growed cultivars of an economically important crop plant, such as tobacco, under suitable growth conditions (Ruiz and Romero, 1998a), applying $CaCl_2$ at two different levels (2.5 and 5 mM). The highest Ca dose (5 mM) lowered foliar levels of P and caused P accumulation in the roots (Ruiz and Romero, 1998a). As bioindicator of quality and uptake of P, the level of non-structural carbohydrates also responded to increments of Ca in the rhizosphere (Hepler and Wayne, 1985). Other authors have observed an accumulation of sugars, particularly starch, in presence of high Ca concentrations (Wei and Sung, 1993). In tobacco, the application of increasing $CaCl_2$ dosages showed that the highest concentrations of glucose, sucrose, fructose and starch in roots were induced by the lowest dosage. In the leaves (Table 1), the highest levels in these sugars were resulted from the highest Ca dose (Ruiz and Romero, 1998a). Thus, the effect of Ca on sugar levels was possibly the most important fact concerning the impact of the Ca on the P translocation. As opposed to the levels of sugars, dry biomass yield remained stable in leaves at the highest $CaCl_2$ dosage (5mM), whereas in roots the dry weight increased with Ca dosages. Both processes were related to the effect of the Ca on the levels of P, which were higher than necessary for optimal growth (Table 1; Ruiz and Romero, 1998a). In these tobacco experiments, the development under growth-chamber con-

Table 1. Effect of Ca ($CaCl_2$) application on carbohydrates and dry matter on root and leaves of tobacco plants.

	Ca treatment (mM)	Glucose (mg g^{-1} f.w.)	Fructose (mg g^{-1} f.w.)	Sucrose (mg g^{-1} f.w.)	Starch (mg g^{-1} f.w.)	Dry matter (g)
Root	2.5	8.4	5.3	6.3	14.1	0.78
	5.0	4.4	2.1	2.9	20.3	1.13
Leaf	2.5	13.4	8.1	9.7	21.6	0.88
	5.0	22.2	12.7	16.7	34.4	0.85

Data extracted from Ruiz and Romero (1998a).

ditions reveals the true effect of the $CaCl_2$ on sugar levels and dry-matter production, as well as the interrelationship between Ca and P in this crop, when the remaining factors and conditions are controlled in an axenic growth medium as the growth chamber.

The application of $CaCl_2$ to field-grown apple trees boosted fruit-quality characteristics, and reduced physiological disorders caused by Ca deficiency (Malakouti et al., 1999). By the way, it should be noted that these trees were grown on high-$CaCO_3$ soil, a condition that may have partly covered the Ca needs of this crop.

Both examples indicate how different environmental condition – absence or excess of Ca in the substrate, plus $CaCl_2$ supplementation – induces similar reactions: enhanced crop quality.

Pesticides

In the example of the previous section, the application of an external agent, in this case $CaCl_2$, increased the crop quality in species as different as apple tree and tobacco plants. Another quite common external agent employed in plant-production techniques is the pesticide application. Once applied to the crop, these substances are degraded by the action of different factors as water, temperature, light, etc., making it difficult to know its complete effects on the plant biochemistry and physiology. We applied the fungicide *Carbendazim*® (Sigma-Aldrich Química S.A., Madrid) on tobacco plants at three different rates: 1.3, 2.6, and 5.2 mM. At the harvest stage, we found greatly increased dry-matter yield at the manufacturer-recommended dosage of 2.6 mM, respect to the control (without application of Carbendazim. This effect was significantly stronger when the foliar application of *Carbendazim* was combined with boron (B as H_3BO_3) at 8 mM, since this micronutrient can elicit the plant-resistance mechanisms to pathogen infections. Using this combined application of micronutrient supply and fungicide, we can reduce the biocide application levels without sacrificing effectiveness (Ruiz et al., 1999). We selected this fungicide based in previous experiments, that revealed a substantial influence of Carbendazim in the metabolism of phenolic and oxidative compounds, which are known to have a key role in plant defensive responses as well as in plant development under diverse conditions of stress, but applied at higher dosage than established could affect not only the dry matter yield but also the foliar pigment and nutrient status, developing in extreme situations phytotoxicity effects in the plant (Garcia et al., 2001).

2.1.2. *Greenhouse experiments*

The rigorous control of environmental conditions in the growth-chamber, being expensive, is not profitable for commercial production. Consequently, despite higher yields than with other techniques, it becomes necessary to use less strict methods. Thus, greenhouse cultivation has become tremendously widespread in the last 20 years, and information is needed concerning the response of the crops to the changes under such agricultural, environmental and physiological conditions. Studies needed include the evaluation of the fertilizer application, irrigation-water quality, and environmental factors. In contrast to the absolute control in the growth chambers, the

conditions used in the greenhouse experiments depend largely on local environmental, sociological and economic variables and even on the building materials of the greenhouse (See Víllora et al., 1998, 1999, and references therein).

Greenhouse production activities usually focused in vegetables, ornamental species, and fruit production. The developmental cycles of the greenhouse-growed plants could be altered by the local environmental factors (temperature, humidity, illumination, soils, etc.) outside the greenhouse. Greenhouse cultivation is widespread throughout Mediterranean Europe, where climate and soils favor early yields, and thus attractive economic benefits for the farmers despite the heavy expenses compared with the cost of other cultivation techniques. While the greenhouse themselves encourage greater yield, specific cropping techniques inside the greenhouses are used to solve specific problems and boost yield even at impressive levels.

Rootstocks
One such technique, also employed in field crops, is the use of rootstocks to induce resistance against soil pathogens (Ashworth, 1985), salinity (Behboudian et al., 1986; Picchioni et al., 1990; Walker et al., 1987), and chlorosis caused by calcareous soils (Bavaresco et al., 1991; Romera et al., 1991; Shi et al., 1993; Sudahono and Rouse, 1994). Rootstocks are used even to alter the foliar content of certain nutrients (Ruiz et al., 1997). Grafting (rootstocks) can improve the plant-substrate relationship. For example, pumpkin rootstocks (*Cucurbita maxima* × *Cucurbita moschata*) of the varieties Shintoza, RS-841 and Kamel were grafted to melon varieties Yuma, Gallicum, Resisto and Arava, using as control non-grafted melon plants (*Cucumis melo* L.) without implanting of the same varieties.

Yield in kg/plant (Table 2) proved higher than in the control, although differences appeared between rootstock varieties with the same scion, but not between different scions. Therefore, the fruit production was significantly affected by the interaction between rootstock and scions, while the scion showed no effect on yield (Ruiz et al., 1997). These results agree with those of Neilsen and Kappel (1996), who indicated that the rootstock increases the yield of the grafted plants by altering the foliar content of different nutrients.

Table 2. Fruit yield in rootstocks of melon varieties (scions) grafted onto pumpkin (rootstocks).

Grafts	Yield (kg plant^{-1})	Grafts	Yield (kg plant^{-1})
Resisto	6.05	Arava	5.16
Shintoza × Resisto	10.20	Shintoza × Arava	8.46
RS-841 × Resisto	9.67	RS-841 × Arava	9.62
Kamel × Resisto	7.86	Kamel × Arava	6.60
Yuma	5.07	Gallicum	5.16
Shintoza × Yuma	9.63	Shintoza × Gallicum	9.65
RS-841 × Yuma	8.44	RS-841 × Gallicum	8.45
Kamel × Yuma	11.30	Kamel × Gallicum	11.4

Extracted from Ruiz et al. (1997).

N-P-K Fertilization

A major factor in greenhouse cultivation is the use of inorganic fertilizers, often exceeding the requirements of a crop for optimal growth. Excess fertilizers, besides increasing salt contamination of the soil and groundwater, reduce yield, lower crop quality, and shorten storage time after harvest, resulting in short-term as well as long-term economic losses. Currently, research is focused on determining the maximum levels that different crops can tolerate without yield and/or quality reduction and with maximum economic return. For example, greenhouse-grown cucumber (*Cucumis sativus* L. cv. Brunex F1) with increasing applications of $N-NO_3$, increased in yield up to a dosage of 20 g/m^2, above which the yield fell by 30% (Ruiz and Romero, 1998b). In fact, there is evidence of the negative influence by which N stimulates vegetative growth to the detriment of the marketable fruit yield (Davenport, 1996).

However, fruit quality (sugars, soluble solids) increased with the N applications, becoming highest at 40 g/m^2 (Ruiz and Romero, 1998b). If in addition to N, potassium is applied, the plant's reaction changes. That is, pepper fruit yield is highest at 18 g/m^2 of N, but with a minimum application of K of 4 g/m^2 (Table 3). Thus, the N rate can be reduced when applying K fertilizer, and the values of commercial yield will surpass those reached with N application alone (Valenzuela and Romero, 1996).

When excessive levels of N-P-K are supplied to eggplants (*Solanum melongena* L. cv. Bonica), although tolerated, depressed fruit yield at N dosages higher than 4 g/m^2 (Table 4), a level substantially lower than that needed by the sweet pepper (*Capsicum annuum* L. cv. Lamuyo) under analogous growth conditions

Table 3. Effect of NxK interaction on biological production (kg/ha) of pepper plant.

		Nitrogen ($g\ m^{-2}$)			
		6	12	18	24
Potassium ($g\ m^{-2}$)	4	42.836	49.927	51.129	47.709
	8	41.024	47.475	48.202	44.882
	12	42.964	45.457	45.378	50.685

Extracted from Valenzuela and Romero (1996).

Table 4. Effect of N, P or K fertilization on yield of eggplant.

	Yield ($kg\ ha^{-1}$)	N ($g\ m^{-2}$)	Yield ($kg\ ha^{-1}$)	P ($g\ m^{-2}$)	Yield ($kg\ ha^{-1}$)	K ($g\ m^{-2}$)	Yield ($kg\ ha^{-1}$)
Control	17,531	4	23,942	13	19,351	5	25,199
		8	16,056	26	20,199	10	20,057
		12	15,085	52	15,428	20	13,942
						30	15,999

Extracted from Víllora et al. (1998).

(Valenzuela and Romero, 1996; Víllora et al., 1998). Meanwhile, the P dosage that induced the highest yield was the 26 g/m² whereas higher application rates reduced yield up to 24% (Table 4; Víllora et al., 1998). As with N, the application of K with greater than 5 g/m² diminished yield up to 45% at 20 g/m², a result similar to that reported by Martin and Liebhardt (1994) for tomato. However, the interaction between fertilizer elements, in this case N and P, applied at low dosages can be positive.

In fact, increased N fertilization that boosts yield enhances the response to the P, thereby producing a positive net interaction. Thus, 15 g/m² of N and 24 g/m² of P gave the highest yield in the eggplant crop (Table 5) when the irrigation water presented a great content of dissolved ions (López-Cantarero et al., 1993).

Ionic stress
Not only the Mediterranean climatic conditions are harsh for many horticultural crops but also there are additional problems of ionic excess in soils originated by the intrusion of salinity in aquifers due to seawater incoming as well as the leaching processes of fertilizers into the groundwater.

This situation translates as increasing salts in the irrigation water, mainly of Cl and Na. As a consequence, the growth, yield and quality are altered in certain crops generally considered sensitive to the salinity. However, not all crops are affected by high levels of Cl and Na in the irrigation water (classified as dangerous water because of its electrical conductivity; C.E. (USSL, 1954). For example, zucchini (*Cucurbita pepo* L. cv. Moschata or *Cucurbita moschata*) were experimentally grown with increasing levels of NaCl in addition to that already present in the irrigation water (Table 6). In this case, we found that 1 g/L of NaCl increased yield, with respect to the lower dosage, and improved firmness, diameter, length, and concentration of soluble solids in fruits (Víllora et al., 1999). Similar improvements were reported in tomato (Satti and López, 1994; Pulupol et al., 1996), and cucumber (Ruiz and Nuez, 1997). When the NaCl levels applied to the zucchini exceeds 1 g/L, yield falls and morphological damages appear in the plants. The total destruction of the crop occurs when the NaCl dosage reaches 9 g/L (Greifenberg et al., 1996). For field experiments, Mitchell et al. (1991) reported that saline irrigation water could significantly improve fruit quality by increasing the total soluble-solids concentration without depressing marketable yield. That is, the NaCl applied in the irrigation water in Mediterranean agricultural areas seriously affects economically important crops. Nevertheless, the use of tolerant or semi-

Table 5. Effect of NxP interaction on fruit production (fruit number/ha) of eggplants.

		Nitrogen (g m⁻²)		
		15	22.5	30
Phosphorus (g m⁻²)	24	285,502	281,270	266,878
	36	253,334	274,285	268,676

Extracted from López-Cantarero et al. (1993).

Table 6. Quality parameters on zucchini fruit under salinity conditions.

NaCl (g L^{-1})	Yield (kg plant^{-1})	Firmness (kg 100 g^{-1})	SSC (%)	Diameter (cm)	Length (cm)
Control	1.04	2.40	4.31	2.55	13.42
0.25	0.91	3.10	4.65	2.77	13.46
0.50	1.02	3.15	4.77	2.70	13.75
1.00	1.45	3.25	5.30	2.79	14.54

Extracted from Víllora et al. (1999).

tolerant cultivars can partly alleviate yield loss, thereby providing economic benefits.

Bioregulators
The use of bioregulators is a common horticultural practice to improve yield (Latimer, 1992). Bioregulators can act on rooting, flowering, fruiting and fruit growth, leaf or fruit abscission, senescence, metabolic processes, and stress resistance (Nickell, 1988). The antiauxin toluipthalamic acid, extended the spring and winter greenhouse production seasons of many plant species, including tomato, potato, and pepper, by promoting fruit set and development (Nickell, 1982),. reduced transplant shock and increased plant yield by applying abscisic acid to bell pepper seedlings immediately before transplanting (Berkowitz and Rabin, 1988). The application of a mixture of growth regulators and nutrients augmented pepper yield and nutrient availability in the fruit (Csizinsky, 1990). Different commercial bioregulators applied under greenhouse conditions induced different responses, independently of the cultivar used. For instance, the application of NAA (naphthalenacetic acid) to grape boosted yield (Reynolds, 1988), while the application of Biozyme (gibberellic acid + indole-3-acetic acid) increased dry-weight production of bean and corn (Campos et al., 1994).

In different pepper cultivars, Biozyme increased yield (Elsayed, 1995), and improved parameters of quality. In the Lamuyo pepper (Table 7), different bioregulators induced varying yield results (Belakbir et al., 1998); the highest yield resulted from the application of NAA and Biozyme, while quality parameters proved highest with the application of GA$_3$ (gibberellic acid; Table 7).

However, the effects of bioregulators are not due exclusively to the chemical composition of the substance, but also to application frequency and dosage (Ruiz et al., 2000).

Genetic variability
As mentioned in the introduction to this chapter, different cultivars vary in their response to the use of any nutrient under different conditions of light, temperature and humidity. For example, genetic variability in nitrogen-use efficiency has been recognized for many years (Smith, 1934). This variability has been partitioned into differences in the uptake and use of N (Pollmer et al., 1979; Reinink et al., 1987). Teyker et al. (1989), by quantifying genetic variation in N-use efficiency

Table 7. Effect of bioregulators (CCC: chlormequat chloride; NAA: naphthaleneacetic acid; GA$_3$: gibberellic acid; Biozyme: GA$_3$+IAA (indole-3-acetic acid)+zeatine+micronutrients) applications on fruit quality parameters in pepper plant.

	Total yield (t ha^{-1})	Firmness (kg)	SSC (%)
Control	44	0.60	0.805
CCC	49	0.57	1.000
NAA	55	0.59	0.880
GA3	47	0.63	0.980
Biozyme	53	0.56	1.020

Extracted from Belakbir et al. (1998).

in corn, demonstrated that selection for increased efficiency was possible. The presence of genotypic variation related to N accumulation and use has also been demonstrated in wheat (Dhugga and Waines, 1989). Thus, the potential exists for developing superior N-efficient cultivars in some crops.

In greenhouse-grown tomato, genotype influenced marketable, non-marketable, and total yield (Table 8). The highest non-marketable yields occurred in G9, G10, and G12, exceeding the levels of G2, G3, G6, and G8 by up to 205%. The G6 plants had the highest levels of marketable yield, surpassing that of G10 by 75%. The highest biological yield was harvested from G6 and the lowest from G5 and G10 (Ruiz and Romero, 1998c). Confirming that N utilization can be a determining factor for yield (Mattson et al., 1991; McDonald et al., 1996), the highest marketable yields were recorded for G2, G3, G6, and G8 (Table 8), the genotypes defined above as having intermediate efficiency in N-utilization (NO$_3$). In contrast, genotypes that were highly efficient in N-utilization (G7, G9, G11, and G12) had low marketable and high non-marketable yields. High N utilization of these genotypes can encourage excessive vegetative growth and less fruiting (Davenport, 1996).

Finally, the genotypes defined as having low efficiency in N utilization (G1, G4, G5, and G10) behaved similarly to those of high efficiency, falling substantially in marketable and total yields. In addition, López-Cantarero et al. (1997), found that heavier N fertilization and therefore increased N utilization by eggplant (*Solanum melongena*) boosted non-marketable yield. These facts indicate the close relationship between yield and N metabolism (Ruiz and Romero, 1998a, 1998b).

The genetic variability affects the fruit quality parameters at the same levels as the final yield. As an example, the carbohydrate content in fruits, one of the parameters which best defines fruit quality (Ho, 1996), showed the higher reservoir in fruit but, depending on the photosynthetic capacity of the leaf.

This reservoir or sink-organ function can be enhanced as soon as carbohydrates are easily conveyed to the fruit. In experiments using different watermelon cultivars (*Citrullus lanatus* [Trumb.] Mansfeld), and similar crop conditions, the foliar accumulation of sugars and pectins differed substantially (Table 9).

Highlighting among the used varieties, 'Perla Negra' plants, in which the levels of both quality parameters surpass those found in the other cultivars (Vargas et al., 1990). In order to apply the carbohydrate metabolism characteristics in a crop-

Table 8. Genotypic variability in relation to tomato fruit yield.

Genotype	Non-marketable	Marketable kg plant^{-1}	Biological
Bufalo	0.93	3.43	4.36
Corindon	0.56	4.21	4.77
Dombelo	0.57	4.14	4.73
GC 773	0.95	3.19	4.14
GC 775	0.67	3.01	3.68
Nancy	0.48	4.62	5.10
Noa	0.94	3.54	4.48
Sarky	0.53	4.31	4.84
Yunke	1.11	3.68	4.79
Volcani	1.04	2.66	3.70
617/83	0.81	3.69	4.50
2084/81	1.46	3.61	5.07

Extracted from Ruiz and Romero (1998c).

Table 9. Genotypic variability in relation to the carbohydrate concentration in watermelon leave.

Genoptype	Sucrose	Fructose	Glucose	Starch (mg g^{-1} d.w.)
Sugar Bell	25.0	6.6	9.0	14.3
Panonia	28.6	7.3	9.6	13.4
Perla-Negra	34.5	10.0	12.9	18.0
Rocio	32.6	8.4	12.2	19.1
Tolerant	31.2	8.5	11.9	22.3
Candida	30.7	8.3	10.9	26.3
Fabiola	22.1	4.8	7.3	22.3
Early-Star	26.9	6.5	9.3	19.0
Carmit	33.9	8.1	12.1	26.6
Resistent	24.2	4.5	7.7	18.5

Extracted from Vargas et al. (1990).

improvement program, an adequate initial range of variation of the character is required, particularity in the direction in which improvement is sought and is also necessary to confirm to what degree the character is inheritable, and the mode of genetic action (i.e. whether it is a dominant or recessive trait, simple or multi-genic, additive or non-additive). The rate of photoassimilate export from source leaves depends upon plant age, leaf age and position, and the rate of carbon fixation (Sánchez et al., 1990). Genotypic variation in the assimilate export has been reported in soybean (Egli and Crafts-Brandner, 1996), tomato (Hewitt et al., 1982), and mango (Tandon and Kalra, 1983). The rate of assimilate transport to the fruit depends upon the fruit's developmental stage, and recent work has demonstrated that the fruits themselves control the rate of import of assimilates. In melon (*Cucumis melo*, L.), the different genotypic varieties studied show broad variability in yield and sugar levels. In this way, the highest yield was reached in the genotype Galia,

while the highest foliar concentrations of sugars were reached in the genotypes Gallicum and Gold-King (Sánchez et al., 1990), consistently under the same greenhouse conditions.

2.2. Experiences under field conditions

By experiments under field conditions, scientists seek to approach the real situations faced by farmers, and thereby achieve higher and better yield in adverse environments and, finally, improve economic benefits. This implies developing new techniques within established crop practices while protecting the environment.

2.2.1. *Season-extending technologies*

Semiforcing' or season-extending technologies using different kind of plastic covers on the soil surface (mulching, mulches), benefits the soil thermal regime, raising soil temperature and moisture, conserving the soil water, avoiding soil erosion, enhancing root growth and bioavailability of nutrients, and ultimately improving crop yield and quality (Decoteau and Friend, 1990; Schmidt and Worthington, 1998). These covers could be made of plastic (e.g. different polyethylene types) or natural materials (e.g. wheat straw or pine straw). The aim is to insulate plants from unfavorable external conditions, encourage early development of the crops either over the whole cycle of development or only during certain developmental phases, for example to avoid autopollinization or pest attack (Hanna et al., 1997, Hanna, 2000).

Plastic mulching
The effect of different colors of plastic material has been studied in different crops, such as strawberry (Albregts and Chandler, 1993) and tomato (Decoteau et al., 1989), and how these material alter the aerial and root-zone temperatures (Ham et al., 1993) and the soil-water content (Mbagwa, 1991). The control of air and root temperatures is one of the main reasons for using plastic mulches. In watermelon cultivation under black or white polyethylene, in contrast to open-air cultivation, white polyethylene reduced the heat accumulation on the surface of the plastic and in the soil at 10 cm deep, with respect to black polyethylene cover from April until July. Thus, clearer plastics can be used to reduce heat accumulation, thereby allowing a longer transplant period for heat-sensitive crops, while the darker plastics would be more appropriate for crops with higher heat requirements (Schmidt and Worthington, 1998). By contrast, organic covers exert opposite effects, as biological decomposition of the material raises ambient temperatures.

For example, a crop sensitive to high temperatures, the potato, is also affected by the color of the polyethylene mulch used, as in the previous experiments, any plastic cover increases root temperatures with respect to open-air plots. However, black polyethylene induces a maximum temperature of 30 °C, reducing tuber production by 4% (Table 10), compared with the yield under coextruded cover (black + white plastic covers), or white (Moreno et al., 1999), and the biomass on a fresh and dry weight of potato plant, also was affected by the root temperature induced

Table 10. Root zone temperature and potato tuber yield under plastic mulches.

Mulch type	Temperature (°C)	Potato tuber yield (kg ha^{-1})
Control (uncovered)	16	41,110
Transparent polyethylene	20	40,020
White polyethylene	23	44,870
Black and white polyethylene	27	45,720
Black polyethylene	30	43,940

Extracted from Moreno et al. (1999).

by mulch (Baghour et al., 2002). When these results are compared with those reported for tomato grown under black plastic and the same crop grown under white-black covers, a similar conclusion is reached – that is, black plastic reduces yield both in ton/ha and in g/fruit with regard to the white-black mulch (Hanna et al., 1997), although root temperatures under both plastics remain lower (± 1 °C) than in the case of the potato (± 3 °C).

While these plastic mulches increased yield with respect to the open-field, transparent polyethylene reduced yield to below that of the open-air plots (Moreno et al., 1999). This decrease was possibly induced by lower temperatures that in turn depressed plant growth and metabolism (Atkinson and Porter, 1996). This contrasts with the results of Ghawi and Battikhi (1988), who attributed lower root temperatures to the shade-effect by the vigorous vegetative growth under the clear cover.

Floating row covers on direct covers
Plastic covers in agriculture are also used to enclose the complete plant, by fixing polyethylene sheets over hoops anchored in the soil. This method is called floating row covers. Due mainly to their ability to trap heat, polyethylene row covers are often used for the production of early vegetables in several regions of the world (Dalrymple, 1973). Slitted or perforated polyethylene row covers eliminate the need for manual ventilation required by solid row covers (Guttormsen, 1972). Such techniques have increased day and night soil and air temperatures in several studies, and have usually increased early crop yield, although total yields have been variable (Taber, 1983; Jenni et al., 1998).

In watermelon, high-density polyethylene row covers inflicted physical damages (puncturing and abrasion) when the covers were removed, causing a reduction in yield. This problem could be minimized by the use of low-density polyethylene cover, without yield losses. Also, the combined application of mulches and row-covers, regardless of the characteristics of the rowcovers, reduce yield. In conclusion, the type of row cover should be appropriate to the crop in order to increase yield without inflicting mechanical damage (Baker et al., 1998).

An example of the application of this technique is the Chinese cabbage (*Brassica pekinensis* (Lour) Rupr.) production under Mediterranean agricultural conditions. This plant is native to areas with high humidity and warmer climatic conditions than in Mediterranean areas, and therefore these types of environments require protec-

Table 11. Influence of thermal regime under row-covers on marketable yield in Chinese cabbage.

Treatments	Air temperature (°C)	Soil temperature (°C)	Yield (kg ha^{-1})
Control (open air)	14.9	20.1	12,797
Polyethylene	20.5	22.9	79,880
Polypropylene	19.2	22.6	74,959

Extracted from Pulgar et al. (1998).

tion such as the row covers. In fact, floating row covers increased production, both biological and commercial, of this plant with regard to a open-air crop (Moreno et al., 2002; Table 11).

Perforated polyethylene and non-woven fleece polypropylene (agrotextile) resulted in up to 5-fold yield increase (Table 11). These data agree with the finding of Loy and Wells (1982), who observed that the polypropylene (agrotextile) provided lower air and root temperatures than did perforated polyethylene, possibly for the high porosity of the material (Centre Technique Interprofessionnel des Fruits et Lêgumes, 1987), but significantly surpassing the open-field yields.

Reductions in nighttime temperatures under the covers, with regard to the external temperatures are reported mainly when the wind is scarce or null and when relative humidity is low (Goldsworthy and Shulman, 1984). Higher temperatures under these covers can reach 30 °C in summer (Jenni et al., 1998). Thus, perforated polyethylene considerably increases production, especially in melon plants (Motsenbocker and Bonano, 1989) and Chinese cabbage (Pulgar et al., 1999), compared with agrotextile covers or no cover at all (Pulgar et al., 2001).

2.2.2. *Deciduous fruit-tree production in southern Spain*

The applicability of different techniques depends fundamentally on the growth habit of the species to be used. That is, not all crops can be covered with organic or plastics materials, as in the case of the fruit trees, although in some areas even these are grown in greenhouses. Therefore trees must be studied mainly in the open-field to improve parameters governing yield and fruit quality (Moreno et al., 1998). Foliar analysis is used to evaluate the nutritional status of fruit trees to identify causes of altered yield, in relation to visual symptoms of nutritional imbalances (Tagliavini et al., 1992). In fruit trees, these kinds of analysis have revealed, on the one hand, yield and fruit quality, and, on the other, the relationships between the foliar levels of several nutrients (Fallahi and Simons, 1996).

However, the foliar analysis of trees presents disadvantages, since accurate results require the standardized collection of samples and the appropriate time of sampling during the biological cycle. In addition, it is necessary to know the agricultural practices in the crop area. In southern Europe, the diversity in species of deciduous fruit trees gives rise to a variety of responses to the same agricultural practice, such as the application of identical fertilization levels. In this sense, for 4 consecutive years, we applied Ca-superphosphate (250 kg/ha), potassium chloride (150

Table 12. Fruit yield (kg/ha) of field grown deciduous fruit trees under Mediterranean conditions during four consecutive years.

Fruit Tree	Species	1992	1993	1994	1995	Average yield
Almond	*Amygdalus communis* L.	2500	3200	2500	3000	2800
Apple	*Malus communis* Poir.	2900	3600	2800	3500	3200
Pear	*Pyrus communis* L.	3600	4400	3500	4200	3925
Pommegranate	*Punica granatum* L.	13200	16500	12900	15700	14575
Hazelnut	*Coryllus avellana* L	5900	7700	7100	5400	6525
Persimmon	*Diospyros kaki* L.	45700	36400	36100	44700	40725
Fig	*Ficus carica* L.	26000	27800	17300	32000	25775

kg/ha), ammonium nitrate (300 kg/ha) and 8000 kg/ha of organic manure to almond, apple, pear, pomegranate, hazelnut, persimmon and fig trees (Table 12), drip irrigated every 15 days. The fruit yield of almond, apple, hazelnut, and pomegranate trees, showed greatest increases at the second year trials, exceeding in all cases averaged value. The average yield in almond tree (1 tree/15m^2 plant density), was 2,800 kg/ha, although at the second-year harvest, yielded 3,200 kg/ha. The apple tree (1 tree/12 m^2) orchards performed better at the second- and fourth-year seasons, both data exceeding the average value. The pear tree (1 tree/12 m^2), showed maximum production in the second year, with 4,400 kg/ha. Meanwhile, for the hazelnut tree (1 tree/2 m^2), averaged 6,500 kg/ha over the four years, and yield was the highest during the second year, surpassing 16% on average.

A characteristic Mediterranean tree, the pomegranate, at 1 tree/11 m^2, yielded a 4-year average of 14,575 kg/ha, also this value was surpassed at the second-year season. Another common Mediterranean fruit-tree, the persimmon, reached highest yield during the first year, 11% higher than the average.

Lastly, the fig tree, of tropical origin but widely cultivated in the Mediterranean basin, yielded 32,000 kg/ha during the last year, this value being far higher than under optimum rates established for highest yield (Moreno et al., 1998). In summary, the fruit yield results of the second year of experiments predominantly gave the highest values, except for the persimmon (the first year), and the fig tree (the fourth year). This confirms, as indicated at the beginning of the chapter, that species or genetic factor has a decisive influence on final yield under the same environmental conditions in the same climate area.

In another kind of experiments, the foliar application of B to apple tree, increased the final yield, but not root-zone B application. In addition, the harvested apples from trees treated before flowering presented high incidences of bitter-pit, internal breakdown, and *Gloeosporium* rot, during the postharvest storage, mostly related to accelerated ripening, induced by the B supply. The physiological usage of B within the apple tree depends on the cultivar (Wójcik et al., 1999).

These examples illustrate that under field conditions, characteristic plants of the agricultural area, or plants well adapted to those environmental conditions are indispensable, especially when these plants present growth habits that prevent the

use of cover materials or greenhouse systems. As shown here, fruit-tree cultivation in a Mediterranean climate does not necessarily require cover techniques, but fertilization applications should be reduced to achieve high yields with better product quality, and to reduce the soil pollution as well as costs of agricultural management.

3. CURRENT STATUS AND FUTURE EFFORTS

In view of the results presented from experiments carried out in growth chambers, greenhouses or the open field, we can deduce that the genetic variability within a species as well as between different species is one of the main variables faced by researchers, particularly in tree crops. It is equally evident that the use of controlled atmospheres for the commercial crops is economically profitable, these techniques can enable species or cultivars to grow in otherwise forbidding areas or during inhospitable seasons. In this way, market demand can be partially regulated, and the agricultural usefulness of an area can be expanded. Next in importance to increasing yield by means of new crop techniques, improving the storage conditions of the products constitutes another focus of the present study. The goal is to maintain as much quality over the storage period, since post-harvest losses can greatly reduce benefits. Such improvements are challenging because not only nutritional alterations take place, but also morphological problems arise. Therefore, the efforts should be directed towards preventing not only of the nutritional disorders caused by cultivation practices or environmental factors during the cultivation, but also some agricultural handling that deteriorates produce quality during harvest, transport and storage. Current efforts in this sense include research in fungicide application to reduce losses during the storage. Also, to avoid chilling injury and its consequent decay, citrus fruit, for example, are intermittently warmed (Wang, 1993) and dipped in hot water (Rodov et al., 1995). In grapefruit, treatments with methyl-jasmonate have also been reported to reduce chilling injury (Meir et al., 1996). Jasmonate appears naturally in the plants as growth regulators in grape, and defends the potato and the tomato from attack by *Phytophtora* and protects barley from mildew (Droby et al., 1999). The success of these techniques varies not only according to the crop species, but also with the post-harvest period of each crop. Besides jasmonate application, other techniques employed with effectiveness are the storage at low temperatures in atmospheres with high levels of CO_2 or low ethylene concentrations. Such treatments seem to be effective for the strawberry storage (Ku et al., 1999), while Ca applications to the harvested fruits discourage fungal decay (Faust, 1989; Janisiewicz et al., 1998). These examples reflect that although the crop processes are vital to good quality and quantity production, the maintenance of this quality through non-aggressive methods for the consumer is one of the current priorities in post-harvest vegetable nutrition.

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PHOSPHORUS MANAGEMENT IN FRENCH BEAN (*PHASEOLUS VULGARIS* L.)

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1. INTRODUCTION

1.1. Cultivation

Beans are a large group of leguminous vegetables that serve as a main source of proteins in human diet. This group comprises several species and some of them are Adzuki bean (*Vigna angularis*); Broad bean (*Vicia faba*); Cluster bean (*Cyamposis tetragonoloba*); French bean (*Phaseolus vulgaris*); Hyacinth bean (*Lablab purpureus*); Lima bean (*Phaseolus lunatus*); Moth bean (*Vigna aconifolia*); Mung bean (*Vigna radiata*); Rice bean (*Vigna umbellata*); Runner bean (*Phaseolus coccineus*); Sword bean (*Canavalia gladiata*); Tepary bean (*Phaseolus antifolius*); Velvet bean (*Mucuna pruriens*) and Winged bean (*Psophocarpus tetragonolobus*). Although all of them are potential sources of protein, all these species are not cultivated in the same region. Depending on the taste and preference of the people each of these is grown and cultivated in different regions across the globe. However, French bean, commonly known as common bean is grown worldwide. Its other names are field bean, kidney bean, pole bean, runner bean, snap bean and string bean. French bean or haricot bean is known by different names in different regions. Adagola, adigura – tsada, ashan guare (Ethiopia); Bab (Hungary); Bohne (Germany); Bonchi Kai (Sri Lanka); Roontje (South Africa); Boontijis (Indonesia); Bo-sa-pe (Myanmar); Bush bean (Rhodesia); Butingi (Philippines); Chilemba (Zambia); Chumbinho opaco (Brazil); Cranberry bean (USA); Edihimba (Uganda); Fagiola (Italy); Fasulia (Sudan); Fasulya (Turkey); Feijao (Portuguese); Frash bean (Indonesia); Harico a couper (France); Haricot nain (Zaire); Icaraota (Venezuela); Ingen (mame) (Japan); Judia (commu m) (Spain); Kanchang bunchis, K. Pendek katjang merah (Malaysia); Porotillo (Peru) (Kay, 1979). It is a species which has many cultivars grown for pods, green seeds or ripe, dry seeds. The distinction between those grown as vegetable and for dry seeds is not clear. Normally it is cultivated in tropics and sub-tropics at an elevation of around 1000 m above mean sea level. It is a delicious, nutritive vegetable consumed when pods are immature, tender and green. Normally the pods are flat or oval for fresh markets but round for processing industry. Beans traded as dried seeds from either this species or of all the other species are generally referred as dried beans.

As per the recent estimates the production of dry beans at global level is 19,393 million metric tons cultivated in an area of 26.603 million hectares with a productivity of 729 kg/ha. Out of this large area, India accounts for 37.52 per cent

contributing to 23.46 per cent of total production. Although India stands first in area and production among Asian countries, the productivity is poor. The productivity in India is 460 kg/ha, which is far behind Lebanon (2400); Azerbaijan (2007); China (1800); Japan (1707); Indonesia (1607) and Iran (1576) amongst Asian countries. Further, the productivity in India is low when compared to productivity (kg/ha) of other continents or sub-continents such as Asia (671); Africa (633); North Central America (1006); South America (736); Europe (1339); Australia (911) and of world (729) (Anon, 1999a). French bean is cultivated in India in an area of 1.48 lakh hectares with a production of 4.2 lakh metric tons (Anon, 1997a).

1.2. Soil

French bean can be successfully cultivated throughout the year in warmer regions of India except in northern parts of India where the weather is severe in both summer (going up to 40 °C) and winter (touching zero). It is sensitive to frost. The fruit set is severely hampered at temperatures above 30 °C. It loves to grow on sandy loams where the soil is loose and root penetration is easy. The desirable pH range of soil for optimum production is 5.5 to 6.8. However Choudhury (1967) opined that optimum pH for F. bean cultivation is pH 5.5 to 6.0 on sandy or sandy loam soils. The crop is too sensitive to water stagnation and to extreme acidic or alkaline soil conditions. The crop quality will be severely affected if grown on problematic soils due to nutrient imbalance. The seed germination is also found affected on heavy soils. Hence loamy soils are preferred for profitable cultivation.

1.3. Season

Among beans all the three groups are noticed, namely long day, short day and day neutral groups. Most of the French bean varieties are day neutral (Choudhury, 1967). Since they are photo insensitive they are more or less cultivated throughout the year. However it is cultivated to a limited extent in high rainfall areas and as well in north India between January and March where the temperature is optimum for the plant growth. It is more suited to the areas having 1000 to 2000 meters above mean sea level receiving rainfall of about 70 cm per annum.

1.4. Nutrients

Although all 16 essential nutrients are required in appropriate proportion for optimum pod yield, NPK nutrients are required in major quantities. It is found that it responds very well to applied nitrogen and phosphates on Indian soils. However it is reported that in UK it responds well to applied N rather than applied P. This may be probably due to variations in nutrient reserves in soils. Further it is reported that a ratio of 3:2:1 for application of NPK may be appropriate based on previous several years experience (Gane et al., 1975).

1.5. Nodulation

Nodulation is seldom seen in F. bean. The nodulation is caused by *Rhizobium phaseoli* in French bean, which is specific to the crop. Due to sparse or little nodulation, the crop depends largely on applied N for optimum pod yields. For this reason the crop responds well to applied nitrogen. Good response has been obtained from as low as 25 kg/ha for Delhi (Arya et al., 1999) to 120 kg/ha in Uttar Pradesh (Singh et al., 1996; Tewari and Singh, 2000). Application of well decomposed farm yard manure encouraged root nodulation. The root development was extensive in FYM applied plants than the fertilizers applied plants (Shivananda et al., 1998). Research work is in progress in India and as well in UK also (Gane et al., 1975) to evolve varieties responsive to root nodulation. There is limited success but more is yet to come.

1.6. Nutritive value

French bean is a nutritive vegetable that supplies protein (24 to 30 percent) and a good source of minerals such as calcium (50); phosphorus (28); iron (1.7) carotene (132) thiamine (0.08) riboflavin (0.06) and vitamin (24) mg/100 g of edible pods (Gopalan et al., 1982). Parthasarathy (1986) analysed edible green pods and found that 100g beans contain 91.4 g moisture, 1.7 g protein, 0.1 g fat, 4.5 g carbohydrates, 1.8 g fiber, 0.5 g minerals. Among minerals it was found that it contained 50 mg calcium, 28 mg phosphorus, 1.7 mg iron, 129 mg potassium, 37 mg sulphur, 4.3 mg sodium and 0.21 mg copper. Also he reported that 100 g beans contain 221 IU vitamin A, 0.08 mg thiamine, 0.06 mg riboflavine, 11.0 mg vitamin C and 0.3 mg nicotinic acid. Further it is reported that French bean is a good source of amino acids such as arginine, histidine, lysine, tryptophane, phenyl alanine, tyrosine, methionine, cysteine, threonine, leucine, isoleucine and valine (Kelley, 1972). For these reasons it is regarded as an important delicious vegetable. The variations in the values reported may be due to reporting from different varieties and sampling at different stages. Hence the values reported from one another are different. French bean is also considered as a medicinal vegetable. The beans are considered anti-diabetic and cure for bladder, burns, cardiac carminative, depurative diarrhoea, diuretic, dropsy, dysentery, eczema emollient, hiccups, itchy, kidney resolvent, rheumatism, sciatica and tenesmus (Duke, 1981).

1.7. Constraints in production

There are several constraints in the production of French bean. These constraints can be classified in to broadly two groups. 1. Lack of varieties suitable to specific soil, climate, export market, process market etc. 2. Lack of appropriate production technology. As a result of these implications the productivity of the crop in India is far below than the anticipated. To increase the productivity of French bean there is a need for coordinated efforts of scientists from various disciplines. Presently the emphasis of the plant breeders is to evolve a variety for higher yields. Since

the crop is susceptible to few diseases such as rust or rot. The breeders are concentrated in evolving a variety resistant/tolerant/less susceptible to the disease. But there is no concern among breeders – plant nutrition scientists combined to evolve a variety for N or P stress conditions. The deficiency of these two nutrients will continue to daunt our productivity of almost all agricultural crops.

The concern for soil health has not gained momentum as much as it has gained with respect to plant health. If the soil health is not cared for, probably plant health deteriorates at much faster rate. Hence there is a need to re-orient strategies to strengthen plant breeding programs to accommodate plant nutrition as priority. Since Indian soils are starved of N and P mainly, there is a need to screen varieties for such soils. Although the genetic base for the crop is limited there is always scope for increasing the genetic base through exchange programs. For this purpose there is a need to develop a data base to be used by plant breeders where all the information is available.

The second biggest problem with respect to production technology is lack of 'appropriate' technology. Although recommendations for nutrients (mostly NPK) have been worked out the quantities have been too large. Re-visiting the problem to reduce the fertilizers is demanding since the crop duration is too short and the economic viability of growers also inhibits to apply such large quantities. The question that needs immediate answer is how to increase the utilization of applied fertilizer? By any technique if the utilization efficiency could be increased, can we reduce the fertilizer input without compromising yield? These are some of the intriguing questions that demand immediate attention of soil scientists/agronomists. In this chapter sincere efforts are made to pool the information on these above issues and relevant points are discussed.

2. VARIETIES

2.1. Origin

It is believed that southern Mexico and Central America are the primary centers of origin while Peru – Bolivia – Ecuador region of American continent is the secondary centre of origin. It is now widely distributed in many parts of tropics, subtropics and temperate regions and is the most important food legume of Latin America and parts of Africa (Kay, 1979). Hence French bean is an introduced vegetable to India. But the crop is so naturalized to the Indian subcontinent that the vegetable is familiar to each and every household in the subcontinent. It is grown for human consumption either for its delicious pods as immature green vegetable or as dry seeds. French bean is cultivated throughout India for its high food value and short duration in nature since it can fit in to mixed cropping, inter cropping, alley cropping, multiple cropping or even multistoried cropping. It is extensively grown in southern parts of India throughout the year but restricted to autumn or spring seasons in northern states of India due to harsh weather conditions.

2.2. Varieties – popular

There are several popular varieties grown in the country. Some of them are exotic, introduced into the country long back while few of them are bred and evolved to suit the local environment. The varieties are selected based on their special characters depending on its yield potential or tolerant to a particular pest or disease or sometimes even to the preference for pod shape, color, taste or other properties. There are about a dozen varieties that are popular in the country and interestingly half of them are exotic that are almost naturalized and have become acclimatized and considered as local cultivars. The list of such popular varieties with their duration and characteristics cultivated in India is listed in Table 1.

2.3. Varieties – local

Cultivars are recommended specific to region or locality depending on various factors such as soil, climate (micro and macro), sun shine hours, rainfall and preference of people in the locality for shape, color and other properties of the pod. The resistance of a variety to pests and diseases in the locality/region is yet another factor for acceptance by the farmers. The variety identified by the breeder is first notified at University level based on three years (minimum) performance. Later the variety is tested at several locations (multi locational trials) within the state or region to be released as a state variety. If the variety qualifies for cultivation at different

Table 1. Varieties of French bean cultivated in India.

Varieties	Duration (days)	Yield potential (Quintal/hectare)	Remarks
Arka Komal	70	90	Pods are green, flat, tender
Bountiful		100–120	Pods are borne in clusters
Contender	50–55 days for first picking	80–95	Tolerant to powdery mildew and mosaic
Jampa		80–85	Highly resistant to wilt and withstands warmer conditions
Kentucky Wonder	60–65	100–120	Stringless, fruiting in clusters
Lakshmi	55–60	120–140	Tolerant to angular leaf spot disease
Pant Anupama	55–60	89	Resistant to angular leaf spot and moderately resistant to mosaic virus
Premier	55–60	75–90	Adapted to late sowing
Pusa Parvati	45–50	80–85	Resistant to mosaic and powdery mildew
Selection EC 57080	55–60	115	Pods are green, stringless, tender, round and fleshy
Selection EC 1080		100	Chocolate colored seeds
SVM-1	65–70 days for first picking	105–125	Resistant to angular leaf spot
VL-Boni-1	45–60	105–115	Round, fleshy, stringless and pale green

Source: Chadha, 2001.

agro-climatic regions, then the variety will be tested at different centers in different states by a coordinated body AICRP-Vegetable (All India Coordinated Research Project on Vegetables Crops, Head Quarters at Varanasi). The variety will be screened first at initial variety trial (IVT) if it has to be qualified as a variety at national/regional level. If the variety succeeds satisfactorily at the tested centers then the variety will be screened at multi-locations in advance variety trial (AVT). Upon successful completion of test parameters in AVT, the variety will be notified and released for general cultivation at national level. Based on above criteria several varieties have been identified for different regions of India, and they are listed in Table 2. These varieties have been successfully cultivated in the regions indicated.

2.4. Varieties – exotic

Technically exotic varieties are those accessions that have been brought into the country legally through germplasm exchange treaty by an authentic organization such as National Bureau of Plant Genetic Resources (NBPGR), New Delhi. But prior to 1986 these regulations were not rigid. Well before few decades few accessions have been introduced into the country and they have adopted and got naturalized. Some of these varieties are 'Top crop' and 'Contender' from USA. 'Giant Stringless' 'BKN-74' are from Sweden. The variety 'Wade' and accessions 'EC 24940' 'EC 74958' and 'EC 30021' are from Russia. Today all these cultivars have been cultivated in commercial scale. 'Kentucky wonder' a pole type variety introduced

Table 2. French bean varieties commercially cultivated in different regions of India.

Sl. No.	Region/State	Varieties recommended	Reference
1.	Jammu and Kashmir	Contender	Samnotra et al. (1998)
2.	Himachal Pradesh	B-6	Negi and Shekhar (1993)
		Him-1	Saini and Negi (1998)
		EC-26392, PBL-M-1	Singh and Singh (1998)
		Sum-1, Kentucky wonder	Thakur et al. (1999)
		Contender	Arya et al. (1999)
3.	Punjab	Pusa Parvaty, Premier	Dhanju et al. (1993)
4.	Delhi	PDR-14, HUR-14,	Ahlawat (1996)
5.	West Bengal	VL-63	Das et al. (1996)
6.	NEH region	Tender, Canadian wonder	Roy and Parthasarthy (1999)
	Meghalaya,	PDR-14	Bhagawati and Bhagabati
	Assam		(1994)
7.	Bihar	PDR-14	Dwivedi et al. (1994)
		Uday	Nandan and Prasad (1998)
8.	Uttar Pradesh	HUR-15	Singh et al. (1996)
		PDR-14	Rana and Singh (1998)
9.	Madhya Pradesh	Rajmal	Dwivedi et al. (1994)
10.	Maharashtra	Waghya	Koli et al. (1996)
11.	Karnataka	Arka Komal	Anjanappa et al. (2000)
12.	Pantnagar, Uttar Pradesh	Pant Anupama, UPF – 627	Shridhar and Ram (1999)
13.	Pune, Maharashtra	HPR-35	Deshpande et al. (1995)

from USA is also a successful variety. The other successful introductions are 'Jampa' from Mexico for Maharashtra region and 'Watex' for Nilgiri Hills region and doing extremely well (Thomas et al., 1983).

2.5. Varieties – global

Varieties that are performing exceedingly well at global level are of interest to the breeder for evolving a suitable variety to the given occasion through exchange of germplasm. Several varieties having special characters such as rich in protein, resistant to salinity, resistant to pests and diseases, mosaic, having excellent cooking quality, best for deep freezing, processing market etc. is listed. The information available on new varieties released recently from China, Russia, Europe and other countries are check-listed in the Table 3. This information is relevant to increase the genetic base particularly to the countries where this vegetable is an introduced crop.

3. PHOSPHORUS NUTRITION

3.1. Phosphates – a limiting nutrient

Amongst all the sixteen essential nutrients phosphorus is the most critical nutrient to tackle with because of its fixation and more so in acid soils of tropics and subtropics. For this reason P deficiency is more common in tropics. The P deficiency symptoms are complex in nature and are exhibited in plant through dwarfed plants with thin stem and shortened internodes. Upper leaves are small and dark green and when the deficiency is severe early defoliation occurs. Further the vegetative period prolongs at the cost of shortened reproductive phase resulting in very low pod yields (Howeler, 1980; Howeler and Medina, 1978). India having predominantly sub-tropical and tropical climate in most of the geographical area, management of phosphorus is considered a key issue. The problem is more complex since the country depends on imports for this nutrient.

In India availability of phosphate fertilizers is a limitation because of limited availability of high quality ore. The Phosphate fertilizers that are manufactured in India are single superphosphate (SSP) and di-ammonium phosphate (DAP). At present a small quantity of low-grade rock phosphate is being mined in Mussorie, Udaipur, Jhabua and Bijawar mines in India. For manufacture of either SSP or DAP the crude rock phosphate ore is imported from elsewhere and it is processed in India. Hence the country is totally dependent on high quality ore from other countries. According to an estimate in 1996–1997, 20.38 lakh tons of rock phosphate was imported at an estimated cost of Rupees 477.35 crores. But the country's domestic consumption was 30 lakh tons in 1996–1997. In 1997–1998 the consumption of phosphatic fertilizers rose to 38 lakh tons. There exists a great gap between supply and demand of DAP that is illustrated in Table 4. For these reasons phosphate is considered a limiting nutrient in India.

French bean responds very well to the applied phosphates on Indian soils since

Table 3. List of French bean varieties that are popular at global level.

Variety	Country/Region	Special character	Reference
Augustynka Atut	Moscow, Russia	Resistant to salinity tolerance	Shevyakova et al. (1994)
Ji Yun 2	Hebei, China	Resistant to mosaic virus	Hao and Guo (1993)
Rosecoco (GLP2) Amy	Nairobi, Kenya	Rosecoco was better than others in salt tolerance	Mugai et al. (2000)
TUC 390 TUC 500	Las Talitas Tucuman, Argentina	Potential varieties of northeast Argentina	Vizgarra et al. (1998)
Femira	Moscow, Russia	New variety released	Bakulina et al. (1997)
Khavskaya	Universainaya Russia	New variety released	Anon. (1997b)
32 varieties studied	France	All varieties described	Anon. (1997c)
Gorna oryakhovitsa –2 Zagortes, Dunavtsi –4 and Skomlya-3	General Toshevo, Bulgaria	Rich in protein	Stoyanova and Milkov (1995)
Dunavtsi –1, Kosten-6, Lozen-1 and Presalv Bayo Victoria Bayo Maderdo flor de Mayo M 348	Celaya, Mexico	Good in cooking quality, hard seeds, long cooking time, good imbibition, no problem in working time	Maldonado et al. (1996)
Starnel Astrel, Masai and Flotille	Rumbeke, Belgium	The best variety for deep freezing. The best for processing market	Vulsteke et al. (1994)
Genotype Arc-1 Genotype Arc-2	Goiania, Brazil	Arcelin, a protein responsible for imparting resistance to the weevil attack	Pereira et al. (1995)
Pinto Americano Pinto Laguna and Bayo Zacatecas	Bermeijillo, Mexico	The earliest crop maturity, highest yield, susceptible to root not and, BCMV and common blight.	Pedroza (1994)
BAT 477 Carioca	Brazil	Very high root density, efficient water absorption	Guimaraes et al. (1996)
Aurie de Bacau	Vidra, Romania	Uniform and stable in production	Munteanu and Faliticeanu (1995)
BAT 477 Carioca and RAB96	Goiania, Brazil	BAT 477 and Carioca are better than RAB 96 for drought tolerance	Guimaraes et al. (1996)
Flor de Mayo Bajio Mantequilla Calpan	Montecillo, Mexico	Very good varieties	Revilla et al. (1994)
IAPAR 44, Millionario, 1732 FT, Taruma Serrano, Sao Jose and Rico 1735	Goiania, Brazil	Among 99 accessions these six were resistant to intermediate reaction of *Fusarium oxysporum* in greenhouse studies	Rava et al. (1996)
Borlotto Type Line 22–89, Line 23–89, Line 4–90 Cannellino Type	Ancona, Italy	Promising lines for industry.	Pirani et al. (1994).

Table 3. List of French bean varieties that are popular at global level.

Variety	Country/Region	Special character	Reference
Novo Jalo	Brazil	A new variety for cultivation	Vieira et al. (1994)
Ouro Branco	Brazil	Resistant to rust (*Uromyces appendiculatus*) and also to angular leaf spot (*Pseudomonas syringae*)	Chagas et al. (1994)
Gan Yan 1	China	Resistance to *Colletotrichum lindemuthianum* and to unfavorable conditions.	Sun et al. (1994)
Negro Cotaxtla 91	Mexico	Resistant to *Uromyces appendiculatus* and tolerant to bean golden mosaic bigeminvirus	Lopez and Rodriguez (1993)
KA Jaidukama	Colombia	Resistant to bean common mosaic polyvirus	Roman and Rios (1994)
Masai Astrel Starnel Tavera Larissa Flotille and Clyde	Belgium	Best cultivar for processing market are Masai Astrel Starnet and Flotille Starnel, the best for deep freezing	Vulsteke et al. (1994)
Negro INIFAP	Mexico	Tolerant to bean golden mosaic bigeminivirus and resistant to rust	Sanchez and Salinas (1993)
Ojo de Cabra 73 Ojo de Cabra Regional Pinto and Nacional Morelos	Mexico		Hernandez and Hernandez (1993)
Kharkovskaya Shtambovaya and Pervomaiskaya	Ukraine Russia		Budennyi and Naumov (1994)
Tustynka and Bor	Lublin, Poland	Least susceptible to pathogenic fungi *C. lindemuthianum, Fusarium oxysporum, F. solani, Botrytis cinerea, R. solani* and *Sclerotinia sclerotiorum*	Pieta (1994)
Decibel, Espada Fesca, ISI 5004, Maxima, Monica, Narbonne, Niki, NR545, NUN9271, Pluto, Rido, Senate, Wav4000 and XPB247	Italy	Recommended for both fresh market and for processing.	Dal and Zami. (1993)

Table 3. List of French bean varieties that are popular at global level.

Variety	Country/Region	Special character	Reference
Chang Bai 7	Jiangsu, China	Good quality with little fiber	Gu and He (1993)
Ji yun 2	Hebei, China	Good quality pod, resistant to mosaic viruses	Hao and Guo (1993)
Edmund, Albion, F8 (6766 X 4238)	Italy	Edmund and Albion among navy type varieties and F8 among brown types were the best suited for Italy brought from UK.	Tei and Fiorentino (1993)
Starozagorska, Cheren, Dobrudzhanski, 7 T′ rnovo 13, Astor	Sofia, Bulgaria	Varieties in cultivation	Zhelev et al. (1992)
Sasi	Masonmagyarovr Hungary	Yielding ability 1207 kg/ha	Kesmarki and Takacs (1993)
Long 87-90028	Hailongiang Province, china	2.25 t/ha yield	Zhang (1992)
Fu san Chamg, Feng	Shadon province, China	16-37-9 t/ha during spring, and 22.9–36.4 t/ha in autumn season with good market quality	Wang (1991)
Cuyano INIA, Burros, Argentinos, Tortoia, Corriente, Flutilla, Corriente	Santiago	Very good varieties for Chile	Tapia et al. (1992)
Jubilatka wanta	Lublin, Poland	Resistant to *Fusarium* spp, *B. cinerea*, *R. solani* and *S. sclerotiorum*	Pieta (1992)
VIKI	Skopje, Yugoslavia	Yielding activity 29 t/ha	Tudzarov (1992)
L207-70, Diacol-catio, Cargamanto, Mocho	Bogata, Colombia	Dwarf forms of Haricot	Arias et al. (1991)
J1 Zhong Yin, Zao Hua Pi	Jilin Province China	Resistant to *C. lindemuthianum* and viruses, yield 15–25.5 t/ha good quality, low fibre content	Wu and Bao (1991)
Rona, Renge, R12 Reka	Kesckemet, Hungary	Resistant to *Pseudomonas phaseolicola*	Velich and Horvath (1990)
Kharkovs kaya 9	Kharkov, Ukrainian SSR	Lodging resistant, 23.5% proteins	Polyanskaya and Zaginailo (1991)

Table 4. Demand and supply scenario of DAP in India (lakh metric tons).

Year	Demand	Supply	Gap
1991–1992	49.5	28.7	20.8
1992–1993	40.5	26.0	14.5
1993–1994	34.8	19.5	15.3
1994–1995	35.9	28.2	7.7
1995–1996	34.2	26.4	7.8
1996–1997	35.5	27.7	7.8
1997–1998	39.0	28.3	10.7

Source: Vikas Singhal 2001.

Indian soils to an extent of 95 per cent are deficient in phosphates. But it is reported that in UK the response of F. bean was poor to applied phosphate possibly due to rich P reserves in soil. The interaction of varieties with soil and the environment may also lead to such varied response. For Indian conditions, there is a need to identify varieties that can respond to low doses of applied P fertilizers rather than high doses. Hence research on identification of efficient genotypes is having lot of importance.

3.2. Rock phosphate as an alternate source

In India, acid soils are present to an extent of 21 m ha. This property of soil can be effectively managed by using rock phosphate. It is well known that rock phosphate reacts with the acid soils to release water soluble phosphate that is available throughout the cropping period. Since rock phosphate is available plenty in the country the same can be effectively utilized. Gajanan et al. (1990) studied the utility of rock phosphate as an alternate source of P fertilizer on the acid soils of Shimoga (pH of soil 5.5), Karnataka. The four treatments were no P, P as rock phosphate, P as single superphosphate and a combination of rock phosphate and superphosphate each in a proportion of 50%. They reported that green pod yield of F. bean was 5.78, 7.24, 6.06 and 6.15 t/ha from the above treatment combinations respectively.

In some cases the soil acidity may not be acidic enough to dissolve and solubilise the rock phosphate to release water soluble P. In such cases the rock phosphate applied to soils may not yield desirable results (pH of soil near 6.0). Then the rock phosphate is acidulated well before it is applied to the soil. Dwivedi (1995) evaluated acidulated products of rockphosphate treated with various mineral acids (nitric acid,, hydrochloric acid, sulphuric acid and ortho-phosphoric acid) at 25, 50, 75 and 100% acidulation. He reported that 25% acidulation with sulphuric acid gave the best results giving the largest bean yield by maintaining higher available P status in soil throughout the growth period and the largest uptake of N and P.

3.3. P requirement of french bean cultivars

P requirement of French bean cultivars vary from region to region based on the P status of soils. Within the same region the recommendations may vary depending on the experimental site. It may be noticed that Thakur et al. (1999) conducted experiments at Palampur, Himachal Pradesh, in varieties Lakshmi, SVM-1 and Kentucky Wonder and reported that 75:64.5:62.3 NPK kg/ha is optimum. But Arya et al. (1999) reported the highest seed yield in cultivar Contender from the same region but probably from different experimental site by application of 25:75:50 NPK kg/ha. These results suggest that Contender demand higher P in the ratio of 1:3:2 NPK compared to 1:0.86:0.83 NPK for three varieties such as Lakshmi, SVM-1 and Kentucky Wonder. Such variations in P requirement have been documented.

It is extremely difficult to determine the limits of P requirement to beans. It is mainly because the essentially minimum quantity of a variety varies largely with the other since it is a genetically inherited character (Fawole et al., 1980). Although essentially it is the second most important nutrient element in importance but it stands sixth in uptake (Howeler, 1980; Howeler and Midina, 1978). Beans absorb nutrients in the following order. N > K > Ca > S > Mg > P.

The minimum P requirement is from Meghalaya (Hilly region) demanding P at 28 kg/ha (Singh et al., 1989) and the highest P at 100 kg/ha from Uttar Pradesh (Baboo et al., 1998) and Bangalore (Thirumalai and Khalak, 1993). Gupta et al. (1996) optimized N:P requirement with water requirement at Varanasi, India. They evaluated three NPK combinations (40:30:20; 80:60:40 and 120:90:60 $N:P_2O_5:K_2O$ kg/ha) at five levels of IW/CPE ratios. They concluded that the highest seed yield (1.51 t/ha) was recorded in the highest application of NPK with 0.75 IW/CPE.

If the soils are polluted with undesirable elements like cement that interferes with soil colloidal properties it would severely limit the soil permeability, aeration and other soil physical and chemical properties. In this connection a study was conducted by Namasivayam (1994) on red soils polluted with cement dust. He found that application of N and P fertilizers applied with anionic polyacrylamide as soil conditioner improved seedling emergence by 63 per cent.

Rana et al. (1998) conducted field trials in Uttar Pradesh during 1991–1993 with four levels of N (0, 40, 80 and 120 kg/ha) and three levels of P (0, 50 and 100 kg/ha). They reported that dry matter production increased upto 120 kg/ha. Increase in seed, dry matter, P content, uptake of N and P was significant upto 100 P_2O_5 kg/ha. Straw dry matter increased upto 50 P_2O_5 kg/ha.

Ahlawat (1996) evaluated several cultivars on the neutral soils of Delhi, India, for their yield and P uptake. He reported that among the cultivars evaluated PDR-14 gave the highest number of pods/plant with bold seeds and the plants were taller than other cultivars. HUR-14 recorded the highest number of seeds/pod. PDR-14 recorded 25.8 and 44.7% higher seed yield than VL-63 and HUR-15. The response to the applied P was linear upto 26.4 kg/ha. P application greatly increased pods/plant and seeds/pod.

Chavan et al. (2000) studied the uptake of NPK in cultivars Contender, Arka Komal and Waghya and found that highest accumulation of P (6.3 kg/ha) was found in seeds in varieties Waghya and Arka Komal. However, they reported the

highest dry matter production (17.2 q/ha), seed protein production (128 kg/ha) and N and K uptake (31.7 and 12.0 kg/ha respectively) in Waghya. They also reported the highest P uptake (8.5 kg/ha) from the highest N rate (50 kg/ha).

There is a great response of French bean cultivars to the applied P in different soils of the country. Several reports are available across the country wherein systematic studies have been conducted and the optimum doses of $N:P_2O_5:K_2O$ have been reported. A compilation of such information is presented in Table 5.

4. EVALUATION OF VARIETIES – CONVENTIONAL METHOD

When the fertilizer input is in demand and the same need to be used efficiently then one should think of varieties which can absorb and utilize efficiently to produce maximum biomass and seed yield to mitigate the problem. While evolving a variety suitable for pest or disease resistance, plant breeders evaluate several varieties/geno-types and then a suitable variety is bred and released. Accordingly varieties that can perform better on low P status soils need to be identified in view of serious limitation of availability of P. Six varieties of French bean were procured from breeders for evaluation of varieties for higher phosphorus use efficiency (PUE). Here the efforts were limited to identify a variety that is efficient in PUE amongst these six varieties (Kasinath, 1997). Some of the efficiency parameter indices of P uptake were harvest index (HI), phosphorus use efficiency (PUE), phosphorus transfer efficiency (PTE), physiological efficiency and agronomic efficiency (AE).

A lot of work has been done in identifying several phosphorus use efficient strains in beans at CIAT. Some of the efficient strains that respond well to the additional

Table 5. Phosphorus requirement of French bean varieties in different regions of India.

Region/State	N: P_2O_5: K_2O (kg/ha)	Reference
Palampur, Himachal Pradesh	75:65:62	Thakur et al. (1999)
	25:75:50	Arya et al. (1999)
Solan, Himachal Pradesh	80 kg P_2O_5/ha	Jasrotia and Sharma (1999)
	67.3:79.5:50	Singh (1987)
Delhi	25 kg P/ha	Ahlawat (1996)
Varanasi UP	120:90:60	Singh et al. (1996)
Faizabad UP	120:60:60	Tewari and Singh (2000)
Lakhaoti UP	120:50:50	Rana et al. (1998)
Bulandshahr UP	120:100	Baboo et al. (1998)
Pantnagar, UP	120:60:40	Rana and Singh (1998)
		Sridhar and Ram (1999)
Samasthipur, Bihar	120:60:40	Singh (1993)
Kalyani, West Bengal	80 kg P_2O_5/ha	Chatterjee and som (1991)
Meghalaya, NEH region	28 kg P/ha	Singh et al. (1989)
Pune, Maharashtra	75 kg P_2O_5/ha	Deshpande et al. (1995)
	50 kg P_2O_5/ha	Wange et al. (1996)
Bangalore, India	90:80:40	Srinivas and Naik (1990)
	62.5:100:75	Thirumalai and Khalak (1993)

P applications are A440, A254, NAG24, A230, A275, A251 and 82PVBZ1771 (Flor and Thung, 1989).

4.1. Mathematical expressions

The following mathematical expressions were used for calculation of various indices wherein ordinary single superphosphate was used in a pot culture trial by Kasinath (1997).

Phosphorus use efficiency (PUE)

$$PUE = \frac{\text{Total dry matter per unit area}}{\text{Total phosphorus uptake per unit area}}$$

Phosphorus transfer efficiency (PTE)

$$PTE(\%) = \frac{\text{Phosphorus uptake in the fruit}}{\text{Phosphorus in the total dry matter}} \times 100$$

Physiological efficiency (PE)

$$PE = \frac{\text{Pod yield per unit area}}{\text{Total P uptake per unit area}}$$

Agronomic efficiency (AE)

$$AE = \frac{\text{Pod yield per unit area}}{\text{Total P applied per unit area}}$$

Harvest index (HI)

$$HI = \frac{\text{Dry weight of pods per unit area}}{\text{Total dry weight per unit area}}$$

The varieties were compared by making use of these expressions for their performance with respect to PUE. The experiment was conducted by applying 100% NPK without cattle manure or with 50% NPK with cattle manure at 20 t/ha in a pot culture trial. The results are presented in Table 6.

4.2. Ranking of genotypes

Results from the above study suggested that there was significant response from six F. bean varieties to levels of NPK with or without cattle manure. The pod yield

Table 6. Evaluation of French bean cultivars for pod yield and P uptake parameters.

Cultivars	Pod yield (fresh weight) (g/pot)	Harvest Index	P uptake (mg/pot)			P uptake efficiency parameters			
			20 DAS	40 DAS	64 DAS	PUE	PTE (%)	PE	AE
100% NPK + no cattle manure									
Arka Komal	233.2	68.5	1.87	10.37	17.71	575	89	3055	216
IIHR-220	112.3	71.1	2.61	12.68	16.29	601	94	1753	112
IIHR-909	141.6	72.1	3.69	15.05	22.94	409	40	1543	142
Tweed wonder	95.3	38.1	2.39	23.13	39.35	349	62	605	95
Pant Anupama	157.5	78.3	1.27	7.51	15.46	684	117	2111	146
Contender	152.3	62.9	1.13	9.13	13.77	753	111	1696	98
50% NPK + cattle manure at 20 t/ha									
Arka Komal	181.7	78.9	1.27	7.86	12.02	892	138	3778	363
IIHR-220	126.8	65.9	1.43	9.46	14.71	636	128	2390	253
IIHR-909	178.2	79.3	1.08	5.66	9.65	941	173	4337	357
Tweed wonder	81.9	43.9	1.51	8.10	10.69	911	148	2047	164
Pant Anupama	122.3	46.8	1.67	6.67	11.75	959	143	2273	245
Contender	96.4	54.3	1.78	8.99	9.41	791	132	2594	193
SEM ±	17.27	10.62	0.16	0.20	0.27	30	0.50	96	0.9
CD @ 0.005	6.37	NS	0.46	0.60	0.80	113	1.5	282	2.7

Source: Kasinath (1997).

was significantly higher to 100% NPK application without cattle manure than at 50% NPK with cattle manure. P uptake in plant was the highest at 64th day in 100% NPK application without cattle manure. However P uptake efficiency parameters such as PUE, PTE, PE or AE were significantly higher in 50% NPK with cattle manure suggesting that P applied was efficiently utilized in 50% NPK with cattle manure than in 100% NPK application without cattle manure.

The response of six varieties was different for different levels of NPK application either with or without cattle manure. The response of six varieties in 100% NPK application without cattle manure could be ranked as below.

Pant Anupama > Arka Komal > IIHR-220 > IIHR-909 > Contender > Tweed Wonder

The response of six varieties in 50% NPK with cattle manure could be ranked as below.

Arka Komal > IIHR-909 > IIHR-220 > Pant Anupama > Contender > Tweed Wonder

5. EVALUATION OF VARIETIES USING TRACER TECHNIQUES

The two main sources of P for the plant growth are soil (native) and fertilizer (applied) sources. It may not be possible to estimate the absorption of P from

these two sources accurately through conventional techniques but it is possible to partition and quantify using tracer techniques. Through indirect methods it is possible to estimate the contribution of fertilizer P with some accuracy by repeating the experiments for 8 to 10 seasons/years. But it demands lot of expertise, careful planning, and uniform experimental conditions. However it is relatively easy and faster to generate more accurate information using tracer techniques. Sometimes double labeling of fertilizers can also be done to monitor the uptake of P from two different labeled sources in the same experiment. Example. Preferential absorption of $KH_2{}^{32}PO_4$ and $K_2H^{33}PO_4$ can be monitored in the same experiment. Some of the utilities of tracer techniques in fertilizer use efficiency trials are listed below.

1. It allows estimating the absorption of P from soil and fertilizer sources.
2. The technique is highly useful when a large number of genotypes/varieties need to be short listed for higher PUE.
3. An agronomist or a plant nutrition specialist can make use of tracers to identify the appropriate method, depth, level, season and forms of fertilizers for the most efficient utilisation of resources.

In the previous section it was concluded that Arka Komal was the superior variety for the low P status soils of Bangalore, Karnataka. The study was conducted by Kasinath (1997) using ordinary single superphosphate. However the authors have conducted another pot culture trial with ten cultivars using ^{32}P single superphosphate.

5.1. Mathematical expressions

The following mathematical expressions (Anon, 1975) were used for calculation of various indices wherein ^{32}P labeled single superphosphate was used in pot culture or field experiments for computation of PUE indices. Some of the expressions used are given.

Specific Activity of P in plant (dpm/mg P)

$$SA = \frac{dpm \text{ of } {}^{32}P/g \text{ dry matter}}{mg \text{ } P/g \text{ dry matter}}$$

Phosphorus derived from fertilizer (Pdff)

$$Pdff\ (\%) = \frac{\text{Specific activity of } {}^{32}P \text{ in the plant}}{\text{Specific activity of } {}^{32}P \text{ in the fertilizer standard}} \times 100$$

Total uptake of P by plant

$$P \text{ uptake} = \frac{\text{Total dry weight of plant (mg or g or kilogram)} \times \text{concentration of P}(\%)}{100}$$

Fertilizer P uptake by plant

Fert. P uptake = Total P uptake × Fraction of Pdff

Soil P uptake by plant

Soil P uptake = Total P uptake by plant – Fertilizer P uptake by plant

Utilisation of applied P (%)

$$\text{Utilisation (\%)} = \frac{\text{Fertilizer P in plant (mg/plant)}}{\text{Amount of P applied through fertilizer (mg/plant)}} \times 100$$

'A' value

$$\text{A value = (mg P added as fertilzer P/100 g soil)} \times \frac{\text{(\%P derived from soil)}}{\text{(\%P derived from fertilzer)}}$$
(mg P/100g soil)

5.2. Levels of P vs response to cultivars

We have applied phosphate at 25 and 100 percent of the recommended level and studied P uptake efficiency parameters. The recommended level of P as P_2O_5 was 80 kg/ha. We have procured the labeled ^{32}P SSP from Bhabha Atomic Research Centre (BARC), Trombay, Mumbai. The results from this study are presented.

Different varieties respond differently to varied levels of P application. We have evaluated ten varieties of French bean at two levels of P (25 and 100 per cent of recommended P) in a pot culture experiment. The soil is highly weathered reddish brown clay loam belonging to Thyamagondlu series (Udic Paleustalf) having a pH os 6.0. The soil was low in organic matter (0.4% organic carbon), low in available N, P and medium in K. The experiment was conducted for duration of 50 days. The crop was sampled before crop maturity owing to less ^{32}P activity in plant samples. Ten French bean varieties were Arka Komal, Contender, Harvester, IIHR-2, Pusa Parvaty, IIHR-909, IIHR-202-4, IIHR-202, UPF-191 and VL-6.

Single superphosphate labeled with ^{32}P was procured from Bhabha Atomic Research Centre, Trombay, Mumbai, having a specific activity of 0.15 mCi/g P. The plant samples were assayed for ^{32}P at harvest. The results computed on dry matter production, pod yield, fertilizer P, soil P and total P uptake, Pdff (%) and other PUE parameters are presented in Tables 7 and 8.

5.2.1. *Response 25% recommended P*

Dry matter production varied from 7.2 to 10.1 g/plant, which was not statistically significant among genotypes. The pod yield differed significantly across genotypes. IIHR-909 recorded the highest and UPF-191 the least. Total phosphorus uptake per plant varied from 19.4 to 27.7 mg/plant. The magnitude of difference in fertliser

uptake was little and it ranged from 2.3 to 2.9 mg/plant. The phosphorus derived from fertilizer (Pdff) ranged from 9.3 to 12.8 per cent. Some of the efficiency parameters such as PER, PTE, AE, PE and A values were computed.

Utilization of applied P ranged from 17.7 (Harvester) to 22.1 (Pusa Parvaty) per cent. However when the experiments were conducted in field, the values ranged from 5.9 to 7.39 per cent (Iyengar and Shivananda, 1988). The root biomass coming in contact with soil is large in a pot culture, thus higher values have been realized. Further the utilization value at 25% recommended P is higher compared to 100% recommended P, since the amount of fixation of P may be greater when large quantity is applied and vice versa. Agronomic efficiency and physiological efficiency were evaluated in all ten varieties and found that the variety Harvester was very poor in these indices.

'A' value concept is used widely for assessing the available nutrient status of nutrient with respect to the standard using labeling technique (Fried, 1964). In this experiment we have determined 'A' values as a measure of available P from different varieties and found that the varieties vary in their soil available P status. From the results (Table 7) it could be noticed that 'A' values are higher for high pod yielding genotypes and lower for low pod yielding genotypes suggesting that there is a possible relationship between these two concepts.

The details are listed in Table 7. The results were computed and ranked using Friedman's test. The results based on ranking are listed below.

Arka Komal > IIHR-909 > IIHR-202 > VL-6 > UPF-191 > IIHR-202-4 > IIHR-2 = Pusa Parvaty > Contender > Harvester

5.2.2. *Response at 100% recommended P*

Similar to that of above experiment another trial was conducted using 100% recommended P using ^{32}P labeled SSP. The results from this experiment suggested that there was no significant difference among genotypes that varied from 7.8 to 10.1 g/plant, but there was significant difference in pod yield (fresh weight basis) ranging from 23.2 (IIHR-202) to 47.3 g/plant (IIHR-2). Total phosphorus uptake ranged from 21.7 to 30.1 mg/plant. Fertilizer P uptake ranged from 6.1 to 9.1 mg/plant which was nearly three folds that of P absorbed at 25% recommended P. Utilisation of P ranged from 11.6 to 16.5 per cent. This was far less compared to 25% recommended P application. From the above experiments we have observed that total P uptake was more or less same either in 25 or 100 per cent recommended P. The absorption of fertilizer P was nearly three folds in 100% P applied plants compared to 25% P applied plants. Accordingly the absorption of soil P was greater in 25% P applied plants than 100% P applied plants. Similarly other factors such as PER, PTE, AE, PE and A values were computed (Table 8).

Phosphorus efficiency ratio (PER) expressed as kg dry weight per Kg N absorbed was more or less similar at either level of applied P (mean of all 10 cultivars) but significant differences were found among genotypes. The highest PER was observed in the cultivars Harvester and I.I.H.R.-202-4 at 25% and 100% recommended P levels respectively. Phosphorus transfer efficiency (PTE) is the transfer of percent P to

Table 7. Pod yield, P uptake, PUE indices and utilization efficiency of applied P at 25% recommended P.

French bean cultivars	Dry matter (g/plant)	Pod yield (g/plant)	25% Recommended P									
			P uptake (mg/plant)			PdfF (%)	PER	PTE	Utilization (%)	AE	PE	A value (mg/plant)
			Fertilizer	Soil	Total							
Arka Komal	9.9	47.8	2.60	22.70	25.30	10.5	394	51	20.3	3645	1889	110
Contender	9.1	49.3	2.50	24.40	26.90	9.3	342	62	18.8	3765	1841	130
Harvester	10.0	19.9	2.30	20.90	23.20	9.7	429	28	17.7	1522	872	120
IIHR-2	9.4	35.2	2.60	23.50	26.10	9.9	374	38	19.9	2556	1359	120
Pusa Parvaty	9.4	27.5	2.90	22.50	25.40	11.4	353	37	22.1	2099	1067	110
IIHR-909	10.1	50.6	2.60	25.00	27.70	9.3	366	50	19.7	3836	1830	130
IIHR-202-4	9.2	29.6	2.70	21.80	24.50	11.1	351	47	20.6	2262	1198	110
IIHR-202	8.1	26.9	2.60	18.80	21.40	12.8	382	42	20.8	2059	1265	90
UPF-191	8.9	26.8	2.70	20.50	23.20	12.2	402	36	20.7	2049	1207	100
VL-6	7.8	31.1	2.30	17.00	19.40	12.3	410	51	17.7	2370	1577	100
CD @ 0.05	NS	13.5	NS	1.70	NS	NS	49	9.0	NS	1027	392	10.2

Table 8. Pod yield, P uptake, P use efficiency indices, and utilization efficiency of applied P at 100% recommended P.

| Cultivars of French bean | Dry matter (g/plant) | Yield (g/plant) | P uptake (mg/pot) | | | 100% Recommended P | | | | | | |
			Fertilizer	Soil	Total	PdfF (%)	PER	PTE	Utilization (%)	AE	PE	A value (mg/pot)
Arka Komal	8.5	33.4	6.10	18.20	24.30	25.3	354	41	11.6	640	1388	156
Contender	8.7	44.0	7.90	19.50	27.40	28.6	317	58	14.9	840	1598	131
Harvester	9.0	14.2	7.20	18.00	25.20	28.7	360	25	13.8	271	561	130
IIHR-2	10.1	47.3	6.50	21.10	27.60	23.4	368	47	12.3	902	1713	173
Pusa Parvaty	9.9	36.3	9.10	21.00	30.10	31.1	332	41	17.4	693	1190	122
IIHR-909	9.5	34.3	7.70	20.60	28.30	27.2	335	41	14.7	655	1201	141
IIHR-202-4	9.6	32.8	8.20	17.20	25.40	32.7	380	37	15.7	627	1286	110
IIHR-202	7.8	23.2	7.20	14.50	21.70	33.3	360	44	13.7	444	1071	106
UPF-191	8.7	24.3	8.70	17.70	26.40	32.8	328	36	16.5	460	913	108
VL-6	8.8	38.6	7.20	19.70	26.90	26.6	329	46	13.8	737	1432	145
CD @ 0.05	NS	13.7	NS	–	NS	NS	NS	8	NS	261	373	21

the edible part, ranged from 28 to 62 percent at lower P level and 25 to 58 percent at higher P level. At both the levels of P application the cultivar contender recorded the highest PTE. Agronomic efficiency expressed as kg edible part produced per kg N applied was the highest at flowering stage by a factor of 6 (mean of all genotypes) compared to 100% recommended P. Genotype I.I.H.R.-909 recorded the highest at 25% recommended P and IIHR-2 at 100% recommended P. Physiological efficiency (PE) was also higher in 25% recommended P pots than 100% recommended P. The cultivar Arka Komal recorded the highest PE at 25% P and IIHR-2 at 100% P. Dahiya et al. (2000) worked out correlation coefficients from 16 quantitative traits in 48 germplasm lines of French bean grown at Hisar and reported that significant positive association exists with many primary branches per plant, pods per plant, clusters per plant and biological yield. But there was negative association between seed yield and seed weight.

These results from the present study suggest that there is a response among French bean cultivars to application of varied levels of P fertilizers. The results from this trial indicated that Arka Komal is a better variety for soils low in available P status and on the other hand IIHR-2 is better for higher P soils. In consideration of these parameters the genotypes were ranked for their performance using Friedman's test. The ranking of genotypes in order of preference are presented below.

IIHR-2 > Pusa Parvaty > Contender > IIHR-202-4 > VL-6 > IIHR-909 > UPF-191 > IIHR-202 > Arka Komal > Harvester

5.2.3. *Depth of placement*

A field trial was conducted to study the effect of depth of placement on dry matter yield, pod yield, total P uptake, Pdff(%), and utilisation of applied P in French bean variety Arka Komal. The study was conducted using ^{32}P labeled single superphosphate procured from BARC, Mumbai. Recommended $N:P_2O_5:K_2O$ fertilizers at 90, 80 and 40 kg/ha respectively were applied. N and K_2O fertilizers were applied as basal in a band at 5 cm depth prior to sowing of seeds below the seed furrow. Along with N and K_2O, SSP was also applied in split application. Few plots received ^{32}P labeled SSP as first split and rest of the plots received ordinary SSP. After 20 days the second split (remaining 50% of P) was given taking care that the plots that received ^{32}P labeled SSP was alternated with ordinary SSP and vice versa. The plant samples were analysed at two stages of plant growth viz., at flowering and at harvest to monitor P uptake, utilization and phosphorus derived from fertilizer and many other related parameters were computed and compared in presented in the Table 9.

Results compiled at flowering stage indicated that band placement of P fertilizers at 15 cm depth resulted in higher dry matter production, total uptake of P, fertilizer P uptake compared to rest of the treatments. The utilization of applied P and A value was also the highest at 15 cm depth. But the fraction of Pdff (%) was the highest derived from 5 cm depth. These results suggest that most of the the root activity is concentrated in 5 cm depth. Within the plant, all the plant parts such as stem, leaf and flower were equally distributed with absorbed fertiliser.

Table 9. Effect of depth of placement and time of application on dry matter, P uptake, utilization and 'A' values at flowering stage in field plot (1.44 m^2).

Treatment	Dry matter (g/plot)	Total P uptake (mg/plot)	Pdff (%)			Fertilizer P uptake (mg/plot)	Utilization of P (%)	'A' value (kg P$_2$O$_5$/ha)
			Stem	Leaves	Flower			
			Depth of placement					
Band placement of the surface	309	742	57.0	45.1	53.3	355	5.91	100
Band placement at 5 cm depth	286	604	67.0	66.6	65.2	402	6.69	46
Band placement at 10 cm depth	309	795	58.6	53.9	51.6	429	7.14	78
Band placement at 15 cm depth	358	936	41.5	54.5	50.1	443	7.39	102
			Time of application					
Application in 2 splits, 1/2 labeled P as basal + 1/2 ordinary P as top dressing	280	734	16.1	19.6	19.9	132	4.41	417
Application in 2 splits, 1/2 ordinary P as basal + 1/2 labeled P as top dressing	214	522	24.1	18.6	17.9	102	3.39	376
SEm±	21.1	86.7	5.06	4.61	3.94	26.4	0.51	–
CD @ 0.05	63.5	261.0	15.3	13.9	11.9	81.0	1.52	–

Source: Iyengar and Shivananda 1988.

Although the highest utilization was observed from 15 cm depth, there was no significant difference among 5, 10 and 15 cm depth placement, but there was significant difference between surface placement and rest of the treatments.

Placement of P fertilizer at 15 cm depth was found to be superior in favoring higher dry matter production, pod yield and total P uptake even at harvest. Fertilizer P uptake, utilization of applied P were the highest from P placed at 5 cm depth. The fraction of Pdff (%) at harvest was nearly 40% compared to 50% at flowering (Table 10).

From these results it was evident that application of P fertilizer at 5 cm depth was significantly superior to surface placement and there was no significant difference among 5, 10 and 15 cm depth. Hence placement of P fertilizer at 5 cm depth was found to be optimum. Since the crop is cultivated throughout the year we have not evaluated PUE in different seasons. We have found that the seasons have great influence on root activity and absorption characteristics in fruit crops such as mango (Kotur et al., 1997), citrus (Iyengar and Shivananda, 1990a) and grape (Iyengar and Shivananda, 1990b).

We have determined the effect of placement on utilization in okra (*Abelmoschus esculentus*) using labeled ^{32}P single superphosphate and found that the placement of fertilizer at 10 or 15 cm depth was useful rather than placement at 5 cm depth. Utilization of P was found to be 12.7, 17.0 and 17.1 per cent when the P fertiliser was placed at 5, 10 or 15 cm depth respectively (Shivananda and Iyengar, 1990). These results suggested that probably okra is having deeper root system than French bean.

5.2.4. *Time of application*

Time of application is very important to achieve higher P use efficiency. In order to evaluate the above concept a field trial was conducted by applying labeled ^{32}P fertilizer. One set of plants were applied with half the amount of labeled P fertilizer as basal and these plants received remaining split as ordinary SSP after 20 days. Similarly other set of plants received first split as ordinary SSP and the second split as ^{32}P labeled fertilizer. This experiment was conducted mainly to determine the fertilizer uptake pattern during first and second split applications of P fertilizer.

The results from the experiment suggested that there was significant difference in dry matter production, pod yield, total P uptake, Pdff (%), fertilizer P uptake and utilization efficiency due to split application at flowering stage of the crop growth (Table 9). Generally the pod yield, dry matter and P uptake reduced with split application of P fertilizer. This indicates that the recommended P fertilizer has to be applied as basal along with N and K_2O fertilizers. The utilization efficiency was also poor with split application. However the soil available P was significantly higher in split application treatments suggesting that there was no limitation of available P to the plants as evidenced by 'A' values. When the recommended fertilizer was applied in full as basal the available P was very low (only one fourth to one tenth compared to split application of P). This gives us evidence that when P fertilizer was applied as split the plant was unable to utilize it fully may be for

Table 10. Effect of depth of placement and time of application on dry matter, P uptake, utilization and 'A' values at harvest in field plot (1.44 m^2).

Treatment	Dry matter (g/plot)	Total P uptake (mg/plot)	Pdff (%)			Fertilizer P uptake (mg/plot)	Utilization of P (%)	'A' value (kg P$_2$O$_5$/ha)
			Stem	Leaves	Flower			
			Depth of placement					
Band placement of the surface	1180	3810	2.55	32.22	34.57	742	12.36	224
Band placement at 5 cm depth	1110	3080	2.21	47.11	52.43	1079	17.97	96
Band placement at 10 cm depth	1090	3430	2.29	41.55	48.23	996	16.60	120
Band placement at 15 cm depth	1240	3460	2.84	36.80	41.73	1052	17.52	156
			Time of application					
Application in 2 splits, 1/2 labeled P as basal + 1/2 ordinary P as top dressing	1100	3390	2.31	14.45	18.19	357	11.90	502
Application in 2 splits, 1/2 ordinary P as basal + 1/2 labeled P as top dressing	830	2220	1.68	13.05	13.81	219	7.27	613
SEm ±	90	290	0.20	3.31	3.24	43.3	0.75	–
CD @ 0.05	NS	870	0.60	9.98	9.77	207	2.26	–

Source: Iyengar and Shivananda 1988.

more than few reasons. Firstly the plant demand was more than the P supply. Secondly, the split application applied after 20 days did not reach the active root zone. Due to practical problems the second split was applied on the surface and only first split was incorporated at 5 cm depth. Due to short duration nature of the crop probably the demand for P is in the initial stages of the crop growth.

The results at harvest also confirmed the findings obtained at flowering. Similar to that of above findings, pod yield, total P uptake, Pdff (%), fertilizer P uptake and utilization percent were significantly low in plants that received split P application. The fraction of Pdff (%) in plants that received P in split was nearly one third to that of plants that received full. Similarly the fertilizer P uptake in split P application plants was one half to that of plants that received full recommended P as basal (Table 10).

Among the split application of P fertilizer, results suggested that the plants that received first split was much better than the plants that received second split. The total P in all the treatment combinations was more or less same but there was variation in fertilizer P indicating that the plants applied with split application explores and forages P from soil source. Hence this clearly indicates that we have to apply the fertilizer near the vicinity of the root zone so that the utilization of the applied fertilizer is increased.

Based on the results it could be concluded that basal application of P for French bean is beneficial rather than split application. Basal application of P fertilizer increased P use efficiency from 7.27 to 12.36 per cent. At flowering stage of the crop the utilization was 3.39 in split application and 5.91 in basal application of P. In split applied plots available P was very high.

5.2.5. *Root activity studies*

Root activity of French bean variety Arka Komal was monitored by application of ^{32}P labeled fertilizer at four depths (surface, 5, 10 or 15 cm depths) in a field trial. The results suggested that the root activity was more or less equally distributed at all depths ranging from 22.6% at surface to 25% at 15 cm depth at the time of flowering. Similar trend was noticed at harvest also. The highest root activity of 28.2% was noticed at 15 cm depth at harvest stage of the crop (Table 11).

Root system in French bean is also an inheritable trait. Root proliferation is given importance since variety with extensive root system will have higher probability

Table 11. Distribution of root activity at various depths in French bean cv Arka Komal.

Method and depth of placement of P fertilizer	Root density distribution (%)		Mean (Percent)
	At flowering	At harvest	
100% basal, surface placement	22.6	23.6	23.1
100% basal, 5 cm depth placement	24.7	23.8	24.3
100% basal, 10 cm depth placement	27.7	28.2	27.9
100% basal,1 5 cm depth placement	25.0	24.3	24.7
CD @ 0.05	2.76	3.13	–

to escape drought and tolerates drought. Zhelev et al. (1992) conducted field trials on six root system traits in six *P. vulgaris* varieties and five mutants and as well two introduced lines. They reported that root system volume ranged from 4 to 11.7 cm^3 and was the greatest in the mutant ML-31. They also reported that there was a correlation between mean weight of the root system and total plant biomass.

We have observed that production of root biomass varies with the form of availability of nutrients. If the nutrients are available easily to the plant in water soluble form (inorganic form) then the production of root biomass decreases. Instead, if the nutrient is in the organic form which is not easily available to the plant then the production of root biomass is higher. Ratio of shoot:root was in the order of 90:10, 77:23 and 68:32 in fertilizer, cattle manure and no fertilizer or no cattle manure applied plants respectively (Shivananda et al., 1998).

Guimaraes et al. (1996) have identified that very high root density can help a variety to be drought tolerant by way of its higher ability to absorb more of water from the surroundings by increasing its (root) surface area. Accordingly they identified BAT 477 Carioca – an efficient variety in water absorption in Brazil.

6. CONCLUSIONS

1. French bean is an important commercial vegetable grown throughout the globe for its delicious green pods or dry seeds. Potential market exists for export-import trade.
2. A great number of varieties are present offering a great deal of heterogeneity for plant breeders to breed tailor make varieties to the given situation. Ample number of varieties is available in India specifically developed to certain agroclimatic regions to suit the local conditions.
3. Across globe a large number of varieties are available with special characteristics to deal biotic and abiotic stresses.
4. Phosphorus nutrition is a problem and needs to be tackled through management techniques. One of the solutions is to identify a plant type that can be grown profitably in P stress conditions. Screening six varieties of French bean to different levels of P suggested that Arka Komal is the best for low P status soils of India.
5. Response of a variety to the applied P can be monitored through conventional and tracer techniques. Tracer techniques are sensitive, reliable and accurate for monitoring utilization efficiency of applied P fertilizers.
6. Studies conducted using ^{32}P labeled fertilizers also indicated that Arka Komal is the variety for low P soils. For higher P status soils IIHR-2 can be selected for better response in terms of dry matter and pod yields.
7. Increasing utilization efficiency of the applied fertilizer is essential to realize higher returns to the every rupee invested. Depth of placement and time of application are the most important factors in achieving higher phosphorus use efficiency.
8. It was observed that placement of fertilizer at 5 cm depth resulted in maximum P utilization efficiency and the least was from surface application. It was also

observed that deeper placement upto 15 cm depth did not increase P utilization efficiency.

9. Application of P fertilizer at the time of sowing the seed as basal is the most useful practice. Application of P fertilizer in splits was evaluated and found that application in split as 50% at the time of sowing and rest 50% after 20 days was found to record 11.90 and 7.27 per cent utilization compared to 12.36% applied as basal.

Distribution of root activity was found to be uniform more or less up to 15 cm depth or below. Maximum root activity of 27.9% was found to be at 10 cm depth.

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NUTRITION AND CALCIUM FERTILIZATION OF APPLE TREES

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1. INTRODUCTION

Calcium (Ca) nutrition of apple trees (*Malus domestica* Borkh.) is attracting increasing interest due to the widespread occurrence of Ca-related disorders. Apple flesh tissues with low Ca concentrations are sensitive to bitter pit, cork spot, cracking, internal breakdown, lenticel breakdown, low temperature breakdown, senescence breakdown, superficial scald, watercore and sunburn (Ferguson et al., 1999; Raese, 1996; Shear, 1975). The occurrence of these disorders, and particularly bitter pit, causes great losses to growers and fruit packing houses. With continued expansion of apple production, the problem of fruit Ca deficiency has been increasing. Economical losses related to production of fruit with low Ca levels result not only from the occurrence of physiological disorders but also pathological diseases (Conway et al., 1994). Apple fruit with low Ca status are sensitive to pathological diseases, even through they are stored in controlled atmosphere storage. Moreover, fruit poor in Ca have low storage potential because Ca plays a critical role in ripening and senescence processes (Marcelle, 1995; Stow, 1993). To avoid or reduce losses related to production of fruit with low Ca status, it is necessary to recognize processes of uptake and transport of this element within the plant.

2. CALCIUM DEMAND OF APPLE TREES

Terblanche et al. (1979) reported that the roots contained 18%, wood 40%, bark 11%, leaves 13% and fruit 18% of the total Ca content of apple tree. The Ca requirement of mature apple tree is remarkably high in comparison to the requirement for other mineral nutrients. With 14-year-old apple trees, Haynes and Goh (1980) found that Ca comprised 80% of the total inorganic nutrient content of the above ground portion and 35% of the total root inorganic nutrient content. At a density of 500 trees per hectare, entire trees contained 84 kg Ca·ha^{-1}, while other macronutrients with sulphur and chlorine combined totaled only 105 kg ha^{-1}. Batjer et al. (1952) showed that mature 'Delicious' apple trees took up 167 kg Ca·ha^{-1} at a density of 124 trees per ha; however, 110 kg Ca returned to soil as fallen flowers, fruitlets and leaves, and as pruned shoots; apple fruit took up 4 kg and leaves up to 86 kg Ca·ha^{-1}. Because of lower production of wood tissues it is assumed that Ca uptake by apple trees planted at high densities is slightly lower compared to those at low densities.

3. STATUS AND CALCIUM FORMS IN SOIL

Calcium is the fifth most plentiful element in the earth's crust with average concentration of 3.6%. In non-calcareous, highly weathering soils, the status of Ca is below 1% whereas in calcareous soils it is usually higher than 5%. Generally, the level of Ca in soil depends on parent material, degree of its weathering, and climatic conditions. Three fairly distinct Ca fractions in soil can be recognized; non-exchangeable Ca, exchangeable Ca and soluble Ca. The non-exhangeable form consists of minerals such as plagioclase, feldspars, augite, hornblende, epidote, Ca sulphates, Ca carbonates and Ca phosphates. Among these minerals, Ca sulphates and Ca carbonates have the greatest solubility. Calcium sulphates usually occur in arid soils, where concentration of sulphate in soil solution exceeds 0.01 mol L^{-1} whereas Ca carbonates are found only in soils with pH above 7.0. At soil pH 7.5 to 8.0, Ca sulphates and Ca carbonates can coexist. The other Ca minerals contain less Ca than Ca sulphates and Ca carbonates, slowly weather and consequently have little importance in supplying Ca to the soil.

In many soils, Ca is the prevailing cation on the exchange complex. Alkaline soils rich in Na, acid soils containing large amounts of H and Al, and serpentine-derived soils high in Mg have cations other than Ca as the dominant exchangeable cation. Exchangeable Ca is in equilibrium with Ca ions in soil solution. Thus, increase or decrease of exchangeable Ca will induce changes in amount of Ca in soil solution. On soils with low cation exchange capacities (CEC), equilibrium between soluble and exchangeable Ca is achieved faster compared to those with high CEC. It is estimated that the amount of exchangeable Ca in soil ranges from 500–2000 mg·kg^{-1}. Calcium concentration in soil solution accounts only for 2–10% of exchangeable Ca. Barber et al. (1962) reported that soluble Ca concentrations in 135 north central U.S. soils ranged from 5 to 100 mg·L^{-1}; the most common values were 20–40 mg·L^{-1}. Reisenauer (1964) showed that levels of soluble Ca in 979 soils (mainly from the western U.S.) ranged from 50 to 1000 mg·L^{-1}, with 54% of the values in the 50 to 100 mg·L^{-1} range. Al Abbas and Barber (1964) obtained soil solution Ca concentrations of 0.6 to 2.3 mg·L^{-1} for acid soils from South Carolina. Labetowicz (1995) reported that status of soluble Ca in soils from Central Poland ranged from 3 up to 168 mg·L^{-1}; however approximately 50% soils had 20–69 mg Ca·L^{-1}. These data indicate that range of Ca concentration in soil solution is wide. It worth noting that with increasing Ca concentrations in soil solutions leaching of this element from top layers of soil increases. It is estimated that in Poland where sandy soils predominant, Ca losses due to leaching into deeper soil layers and/or into ground waters are 300–600 kg·ha^{-1} annually. Large Ca losses take place particularly on sandy soils with low CEC, in the second year after liming, and under conditions of high rainfalls in spring and autumn when activity of roots is reduced.

4. CALCIUM UPTAKE

Uptake process of mineral nutrients by root system consists of four stages: (i) release of ions from soil surface into soil solution, (ii) movement of ions in soil solution

to the root surface, (iii) adsorption of ions by cell walls of roots, and (iv) movement of ions across a cell membrane.

Release of exchangeable Ca into soil solution depends on saturation of exchange complex by Ca ions, type of soil colloid and the presence of other cations adsorbed on exchange complex. With increasing saturation of exchange complex by Ca ions, release of this element into soil solution increases. Thus, on regularly limed course-textured soils having natural low CEC, release of Ca into soil solution is particularly high. Soils containing a 2:1 clay (for example montmorillonite) requires a higher saturation to provide Ca supply equivalent to a 1:1 clay (for example kaolinite, illite). Therefore on fine-textured soil rich in a 2:1 clay, applied Ca rates should be high to achieve adequate Ca level in soil solution. In the presence of some cations in soil solution, release of Ca from exchange complex can undergo change. In practice, the largest effect on release of Ca from exchange complex have Al ions. As concentration of Al in soil solution enhances, release of Ca from exchange complex increases. High status of Al in soil solution occurs at soil pH below 5.5. Thus, on acid soils, saturation of exchange complex by Ca ions is relatively low. Calcium moves in soil solution to the root surface by mass flow and/or *via* diffusion (movement of ions along a concentration gradient). The amount of nutrient supplied by mass flow is influenced by the rate of plant transpiration and concentration of ion in soil solution. With increasing transpiration and concentration of Ca in soil solution, movement of Ca to root surface rises. At high rates of transpiration, most Ca in soil solution is moved to root surface by mass flow. When mass flow exceeds the rate of Ca uptake, accumulation of Ca around the roots occurs. Under conditions of low rate of transpiration, the contribution of diffusion process to Ca movement in soil solution to root surface gradually increases. Thus, in spring when transpiration rate of apple trees is low, diffusion is critical process determining movement of Ca to roots. In summer, when most of leaves of apple trees are fully developed, Ca ions are transported in soil solution mainly by mass flow.

After reaching the root surface, Ca is adsorbed to negatively charged exchange sites of cell walls or it moves directly to membrane surface with soil solution. It is still not firmly established whether Ca uptake is a metabolically active, energy-dependent operation or a passive process. In some cases, the presence of respiratory inhibitors and low root temperatures can depress (Iserman, 1970), or have no effect on rate of Ca absorption by plants (Tromp, 1978). According to Maas (1969) character of Ca uptake depends on concentration of Ca in soil solution; in the low concentration range, uptake of Ca is metabolically controlled, whereas at higher concentrations diffusion is the main process responsible for Ca absorption. Based on the electro-chemical approach of ion transport through membranes the comparison between the Ca concentrations and electrical potential inside and outside of the cells an efflux pump for Ca would even be necessary for the maintenance of the relatively low Ca ion concentration inside of the cells (Higinbotham et al., 1967). Presumably the well known action of Ca in decreasing membrane permeability restricts its own permeation into cell.

5. RADIAL TRANSPORT OF CALCIUM ACROSS ROOT

There are two pathways of movement of ions and water across the cortex towards the stele; one passing through the apoplasm (cell walls and intercellular spaces) and another passing from cell to cell in the symplasm through the plasmodesmata. Calcium moves across the root cortex by diffusion, by displacement exchange in the free space, or by a combination of these processes (Bangerth, 1979). Movement of Ca from the cortex into the stele and xylem vessels is restricted by the suberized Casparian strip of the endodermis. This strip has hydrophobic properties and completely surrounds each cell of the endodermis. Thus, to continue past this point, Ca ions must move through the symplast passing through membranes along a cytoplasmic continuum. Once in the stele, Ca ions may enter the xylem vessels through active secretion by xylem parenchyma cells (Biddulph, 1967) or by passive leakage into the vessels (Bowling, 1973).

6. LONG-DISTANCE CALCIUM TRANSPORT

The long-distance transport of water and solutes (mineral elements and low-molecular-weight organic compounds) takes place in the vascular system of xylem and phloem. The overall pattern of Ca movement in woody plants is similar to that in herbaceous plants. Long-distance transport from roots to above ground parts of plants occurs predominantly in the nonliving xylem vessels. Xylem transport is driven by the gradient in hydrostatic pressure (root pressure) and by the gradient in the water potential. Shear and Faust (1970) showed that the transport of Ca in the xylem of young apple seedlings was relatively slow, requiring about 3 days to travel 30 cm. Branfield (1975) reported that Ca concentration in the xylem sap was related to development stage of apple trees; at the stage of bud burst, Ca level in the xylem sap rapidly rose and was maintained to mouse-ear stage and then fell gradually. In the xylem, Ca is adsorbed on negatively charged exchange sites present on the vessel walls. Thus, Ca in the xylem is moved upward in the transpirational stream by a series of exchange reactions. The mobility of Ca in the xylem is increased by the presence of other divalent cations (mainly Mg) which compete with Ca ions for the exchange sites. Also the presence of chelating compounds such as malic and citric acids increases the rate and extent of Ca movement in the xylem (Millikan and Hanger, 1965). If the cation exchange complex of the xylem tissue is saturated or if Ca is chelated, rate Ca movement in the xylem is closely related to transpiration rate (Van der Geijn et al., 1979).

Although, movement of Ca in the xylem is major pathway, there are reports suggesting that the phloem is also involved in the long-distance transport of Ca in apple trees (Stebbins and Dewey, 1972). Faust and Shear (1973) found that Ca applied to the roots was transported at a very slow rate through the phloem to the young developing leaves. Marchner (1995) thinks that Ca transport in the phloem have not significant importance because of low concentration of this element in the phloem sap ranging from trace to 100 µg·ml^{-1}. For comparison, K concentra-

tion in the phloem exudates range usually from 2000 to 4000 µg·ml^{-1}. Moreover, it is believed that low mobility of Ca in the phloem results from forming insoluble Ca phosphates because pH of the phloem sap ranges from 7.0 to 8.0 and phosphate status in the phloem exudates is usually high. According to Zimmerman (1960) low mobility of Ca in the phloem may be also caused by deposition of Ca oxalate crystals in cells surrounding the phloem. The other reason of low mobility of Ca in the phloem may be existence of a Ca specific efflux pump in the membranes of the phloem vessels or preferential accumulation of Ca in the cells surrounding the phloem (Marschner, 1974). Regardless of reasons of low Ca mobility in the phloem, it is worth noting that ability to immobilize Ca is dependent on species and cultivar. It seems that apple trees have relatively high capacity to transport Ca in the phloem as was suggested by Faust and Shear (1973).

7. CALCIUM ACCUMULATION INTO LEAF AND FRUIT TISSUES

Calcium moves into apple leaves throughout growing season because transpiration process occurs continuously regardless of stage of leaf development. Old leaves contain more Ca compared to young ones. In mature leaves, Ca is accumulated mainly in the veins as Ca oxalate crystals. In young leaves, Ca is evenly distributed.

It is a long-standing theory that most Ca move into the fruit in the xylem with the flow of water. This occurs predominantly in the early stages of fruit growth when a high surface to volume ratio exists and transpiration from the fruit surface still provides sufficient motive force for water to flow into the fruit. As fruit expand, this ratio becomes less favorable, xylem transport is supposed to decline, and the phloem assumes greater importance in providing both water and minerals. Wilkinson (1968) distinguished 2 phases of Ca accumulation into apple fruit. The first phase characterizes a rapid increase of fruit Ca and lasts during of cell divisions (5–6 weeks after petal fall). In the second phase, Ca uptake either continues at a slower rate, ceases altogether, or Ca is exported back into the tree during dry seasons. The second phase is associated with the period of cell expansion. Quinlan (1969) found that during the first 6 weeks of fruit growth up to 90% of the total fruit Ca was accumulated. However, in many experiments it was reported that Ca was intensively moved into the fruit over the entire growing period (Tomala et al., 1989; Tromp, 1975; Wojcik and Cieslinski, 1997). This would imply that the xylem supply continues to be important over a longer period of growth, or else substantial movement of Ca occurs in the phloem, or other factors are involved in the input of Ca into the fruit. Tromp (1975) accounts for the varying pattern of Ca input into the fruit by suggesting that environmental influences on rate of fruit growth affect the balance between xylem and phloem supply. For example, high air temperatures increase fruit growth rate but Ca influx into the fruit is reduced since an increased growth rate is associated with greater phloem supply. Ford (1979) found that imposition of lower daytime temperatures in the early stages of fruit growth reduced Ca concentrations, mainly by increasing the final fruit weight, but also by effecting a slightly

lower Ca input. Regardless of rate of Ca into the fruit, the rate of expansion of the fruit is greater than the rate of Ca input, resulting in a dilution of Ca in fruit flesh. Therefore, apple fruit are generally poor in Ca.

8. CALCIUM DISTRIBUTION IN THE FRUIT

After 2–3 weeks of fruit growth, there are any considerable differences in Ca concentration between fruit tissues. As the season progresses, concentration gradients develop within the fruit with Ca being highest in the skin, lowest in the flesh, and intermediate in the core (Ferguson and Watkins, 1989). In the cortex, the lowest Ca concentrations occur in its outer zones with an increasing gradient towards the fruit core. Calcium concentrations have also been found to decrease in the cortex and the core from the stem to the calyx end (Lewis and Martin, 1973; Tomala, 1999). This gradient appears to be established by the middle of the growth period, remaining unchanged through to maturity (Lewis, 1980). The blushed side of the fruit may have higher Ca concentration in the flesh than the fruit side without red color (Tomala, 1999). Concentrations of Ca in some fruit tissues is also related to fruit storage. Terblanche et al. (1979) reported increase in Ca concentration (on a fresh weight basis) in the outermost 2 mm of flesh over the first seven weeks of 'Golden Delicious' apple storage. Similar, but less changes were observed in deeper zones of the cortex. These changes probably resulted from Ca migration from the core to the cortex.

9. FACTORS INFLUENCING CALCIUM NUTRITION

9.1. Soil factors

The absolute concentration of Ca in soil solution is less important in controlling Ca uptake than the relationship of Ca to the total salt concentration and its proportionate concentration to that of other ions in solution (Shear, 1975). Along with the ratio of Ca to total salt concentration, specific ions in soil solution may inhibit its uptake. It is well known that absorption of Ca may be depressed by NH_4, K and Mg ions. This negative effect on rate of Ca uptake is more pronounced when concentrations of above-mentioned cations in soil solution are high and Ca level is relatively low. Thus, high rates of $N-NH_4$-, K- and Mg-fertilizers applied on acid soils will reduce the rate of Ca absorption by plants. According to Kotze (1979) the greatest effect on reduction of Ca uptake have NH_4 and Al ions. Therefore, on acid sand with high Al concentrations in soil solution, NH_4-N fertilizers should not be applied in apple orchards. Calcium uptake may also be stimulated by the synergistic effect of other ions in soil solution such as NO_3 and HPO_4 or H_2PO_4 (Jakobsen, 1979). Because NO_3 concentration in soil solution is usually higher compared to HPO_4 or H_2PO_4, it seems that NO_3 ions have a greatest effect on stimulation of Ca absorption. However, it is worth noting that $N-NO_3$ stimulates not only uptake of Ca but also other cations (Kirby and Mengel, 1967).

Under field conditions, soil water deficiency is critical factor reducing absorption of nutrients by plants. Decrease in Ca uptake as a result of low soil moisture is related to the fact that Ca moves in soil solution mainly by mass flow. Moreover, low water content in soil results in increase of total salt concentration in soil solution, which additionally decreases rate of Ca uptake. Slowik (1979) showed that 'McIntosh' apple trees grown in zones with high rainfalls during the growing seasons had higher Ca levels in leaves compared to ones from zone with poor precipitations. Goode and Ingram (1971) in an irrigation experiment with 'Cox's Orange Pippin' apple trees showed lower concentration of fruit Ca with decreased soil moisture. Thus, on sandy soils in seasons with low rainfalls, irrigation may improve Ca nutrition of apple trees.

9.2. Biological factors

Young, newly formed roots tend to take up the most Ca. Thus, absorption of Ca will be the highest under conditions of active root growth. With increasing distance from the tips, Ca uptake by apple tree roots declines rapidly (Clarkson and Sanderson, 1971).

Atkinson and Wilson (1980) have showed that mature 'Golden Delicious' apple/M.9 and Worcester apple/MM.104 had two peaks of root growth during the growing season; one peak occurred in late spring and a second one in midsummer. These authors proved also that newly planted apple trees had different pattern of seasonal root growth compared to mature trees. When trees were young, root growth and shoot growth occurred simultaneously. After three years, the main peak of root growth did not begin until the rate of shoot growth had started to decrease.

According to many authors, uptake of nutrients by apple trees is influenced by rootstocks. Skrzynski (1998) showed that P2 and P22 rootstocks had higher ability to take up Ca than P60 and M.26. In this experiment, P14 and M.9 rootstocks took up the least Ca. Fallahi et al. (1984) reported that leaves of 'Starkspur Golden Delicious' on OAR-1 rootstock had significantly lower Ca concentrations compared to those on M.7, MM.106, and M.1. Granger and Looney (1983) showed that one-year-old apple trees on M.26 accumulated more Ca in leaves than those on M.7, MM.106 or MM.111. Recently, Fallahi et al. (2001) showed that 'BC-2 Fuji' leaves on B9 rootstock had higher Ca status than those on Ottawa 3 and M.7 EMLA.

Head (1969) reported that fruiting of apple trees reduced a rate of root growth; this effect was observed even at light fruit load. In this experiment, heavy fruiting might even eliminate the growth peak found in de-blossomed trees in July through September. The negative effect of cropping on root growth and consequently on Ca uptake is particularly pronounced on apple trees grafted on dwarf rootstocks (Avery, 1970). Pruning of apple trees may also affect root growth. Head (1967) showed that severe dormant pruning stimulated shoot growth and reduced root growth during summer. Therefore, in apple orchards with severe Ca deficiency in fruit, pruning should be performed 1–2 weeks before flowering. Calcium uptake may also be influenced by planting density. With increasing planting densities, uptake of water and Ca per root length unit usually decreases. This is caused by overlapping of the depletion zones of individual roots and reflects interroot competition

for Ca. The abundance of newly formed roots depends also on physical conditions of soil. Generally, a higher number of small roots usually is found in porous soils. Thus, on soils with high bulk density having reduced porosity (macropores >30 μm), absorption of Ca may be limited.

It seems that the status of Ca in fruit is cultivar dependent. Under Polish conditions, 'Jonagold', 'Szampion' and 'Gala' apples have usually lower Ca levels (150–350 mg·kg^{-1} dry weight) compared to 'Idared' and 'Lobo' fruit (500–700 mg·kg^{-1} dry weight). Consequently, 'Idared' and 'Lobo' fruit have high storability even when they are stored in a cold storage. Concentration of Ca in fruit flesh is also associated with fruit size and the number of seeds. Large fruit usually have low Ca concentrations in flesh tissues. Therefore, heavy thinning, severe winter pruning and spring frost decrease fruit Ca as a result of increase in fruit size. The fruit with higher number of seeds are usually rich in Ca which probably is caused increased production of auxins in fruitlets. The number of seeds in the fruit depends mainly on weather conditions during flowering and the presence of bees in this period. If unfavorable conditions to pollination occur during flowering (heavy rainfalls and/or low temperatures), fruit are poor in seeds and consequently in Ca. The number of seeds is also cultivar dependent. Generally, 'Lobo', 'Idared' and 'Elstar' fruit having high number of seeds are rich in Ca. Status of Ca in fruit depends also on fruit position on tree. The fruit from the upper regions of trees have lower Ca concentrations compared to those from the bottom ones. Lower concentrations of Ca in fruit from upper zone of the canopy is caused not only by larger fruit size but also by decreased Ca accumulation into these fruit. Consequently, the fruit from tree top are usually more mature at harvest than those from the bottom of the canopy. To obtain fruit with high storability, harvest should be performed many times in season beginning from fruit from upper tree regions. Status of Ca in fruit is also related to position of fruit on the spur. Central fruit on a spur have higher Ca concentrations than lateral fruit. However, when fruit are thinned to one per spur, central and lateral fruit have similar Ca concentrations. This indicates that competition for Ca rather than position per se is critical factor in Ca accumulation into fruit. An important role in accumulation of Ca into fruit plays spur leaves (Wojcik and Mika, 1998a; Volz et al., 1996). As transpiration rate of spur leaves increases, movement of Ca into fruit enhances. It is suggested that this phenomenon is related to diffusion of Ca from spur leaves to the fruit. The effect of spur and bourse leaves on rate of Ca accumulation into the fruit is particularly pronounced in the early stages of fruit development. Another factor influencing fruit Ca level is crop load. It is well known that fruit from lightly-cropping trees have low Ca levels. This phenomenon is observed regardless of fruit size. Low Ca concentrations in fruit from lightly-cropping trees results not only from increased fruit size but also from strong competition for Ca between leaves and fruit; leaf tissues have higher capacity to accumulate Ca than fruit tissues. Therefore, on young trees or in seasons with low tree cropping, fruit are particularly sensitivity to Ca-related physiological disorders.

10. TREATMENTS INCREASING FRUIT CALCIUM

10.1. Soil management and balanced fertilization

Adequate soil pH, optimum status of available Ca, N, K, Mg, and B, and moderate soil moisture are crucial in increasing fruit flesh Ca. For apple trees, adequate pH is 5.5–6.0 and 6.0–6.5 on coarse and fine-textured soils, respectively. At these pH values, status of Ca in soil solution is high and physic-chemical properties of these soils are adequate for root growth. However, soil pH is dramatically changed by some management practices; this effect is particularly pronounced on coarse-textured soils. Therefore, once per 3–4 seasons, liming should be applied to modify soil pH. It is worth noting that excess liming has a negative effect on availability of P and mostly microelements. Thus, rates of Ca materials must be applied closely according to the needs. On soils with optimum pH, gypsum (Ca sulphate) application may be beneficial in increasing fruit Ca. Increase of Ca in fruit as a result of soil gypsum application is not high but in many cases it is sufficient to reduce bitter pit and senescent breakdown. Response to gypsum treatment is slow, requiring two to four years before an effect appears in the fruit. However, once a response appears, it persists for many years. Ten to 15 kg of gypsum per tree is sufficient to increase fruit Ca. This application apparently does not need to be repeated for at least six seasons. Gypsum application is recommended only on soils rich in Mg because it decreases Mg availability to plants. It is worth noting that gypsum application does not overcoming fruit Ca deficiency, because its effect is too slow and small.

Regardless of source of N, excessive N fertilization decreases fruit Ca status. It is caused by strong competition between one-year-old shoots and fruit for Ca. Under Polish conditions, recommended rates of N in apple orchards usually range from 20 to 100 kg ha^{-1}. Lower given N rates are applied only on soils rich in organic matter (>2.5% C_{org}) and higher rates of N can be recommended on soils having low reserves of organic N (<1% C_{org}). However, N applied at rates above 100 kg ha^{-1} may increase the risk of the incidence of Ca-related physiological disorders. Effect of N fertilization on fruit Ca status depends not only on N rate but also on timing of its application. Nitrogen fertilizers applied during the dormant season strongly stimulates one-year-old shoot growth which consequently decreases accumulation of Ca into fruit tissues. Therefore, many apple growers in Poland apply N fertilizers in the summer (4–5 weeks after petal fall). However, summer N fertilization should be applied only in regions where cold injury do not occur and in the case of growing red apple varieties. This is due to the fact that increased availability of N in the summer delays maturation of buds and woody tissues, and reduces blush development on fruit surface. For many years, N fertilizers have being also applied by Polish growers in the autumn after harvest. At present, application in the fall is not recommended in integrated apple production because this treatment increases the risk of leaching of N beyond the root zone into ground waters. Fruit Ca status depends also on applied N form. N-NH$_4$ fertilizers decrease fruit Ca compared to those with N-NO$_3$. Therefore, Ca nitrate or ammonium nitrate as N sources should be applied in apple orchards where problem of Ca deficiency occurs

frequently. Influence of N form on fruit Ca level is particularly pronounced when high N rates are applied.

Excessive applications of K and Mg suppress uptake and Ca accumulation into fruit. The optimal levels of K and Mg in apple leaves to obtain high productivity and high crop quality are 1.0–1.5% and 0.22–0.32%, respectively. If the status of leaf K is optimal, rates of this element range from 60 to 90 kg·ha^{-1} annually. In Poland, Mg fertilization of apple orchards is not recommended under conditions of optimal Mg level in leaves. When leaf Mg drops below 0.22% and soil analysis confirms its too low level, Mg fertilization is necessary. Thus, the best practical approach for developing K and Mg fertilization programs is leaf analysis in conjunction with soil test. Therefore, to avoid problems with production of fruit with low Ca concentrations, apple growers should make systematically analysis of soil and leaves. In this way, the risk of fruit Ca deficiency induced by over fertilization with K and Mg is strongly reduced.

In Poland, approximately 70% of soils have low B levels. Therefore, in many apple orchards, B deficiency is often observed, particularly in seasons with low rainfalls. In apple orchards, B deficiency often occurs together with fruit Ca deficiency, because B stimulates both uptake and Ca accumulation into fruit. Therefore, on soils with low hot-water extractable B concentrations (<0.3 mg·kg^{-1}), B fertilization is necessary. In the case of moderate B shortage in soil, three foliar sprays of this element should be applied: at the beginning flowering, at petal fall and 2 weeks after later. When soil B deficiency is severe, soil B application at a rate 2–5 kg·ha^{-1} per three years is recommended.

Soil moisture is a fundamental factor in Ca management. Low water level in soil drastically decreases uptake of Ca by plants. Moreover water stress may lower fruit Ca by drawing Ca from the fruit to leaf tissues. Therefore, it is important to irrigate apple orchards on soils with low water retention. Irrigation of apple orchards is particularly necessary in regions with low rainfalls (below 500 mm annually). It is estimated that in Poland about 30% of the total apple orchards should be irrigated to avoid water stress. Presently, only 10% of apple orchards is irrigated. We assume that under Polish conditions maintaining moderate soil moisture throughout growing season, and particularly in the summer, can be critical factor limiting problem of fruit Ca deficiency.

In Southern Poland where soils rich in organic matter predominate, fruit Ca deficiency is caused mainly by intensive shoot growth. To reduce excessive shoot growth and consequently to increase fruit Ca, many apple growers from this region delay herbicide application until after bloom, allow ground cover or weed competition to come in during late summer or sow a cover crop in the herbicide-treated strips.

10.2. Tree management

Tree pruning affects fruit Ca status significantly (Wojcik, 1997). Excessive dormant pruning stimulates vegetative growth and consequently induces fruit Ca deficiency. Moderate pruning just before flowering and thinning cuts, rather than heading cuts, tend to minimize the stimulatory effect of pruning on tree vigor. When pruning

is performed in late summer, fruit have usually increased Ca status. This is caused by lowering shoot/fruit ratio which increases Ca accumulation into fruit. Beneficial effect of late summer pruning on fruit Ca level is found when this treatment is done 4–6 weeks before harvest. Later pruning has small or has no influence on fruit Ca status. A good way to increase fruit Ca is also root pruning. However, positive effect of this measure on fruit Ca level is observed only under some conditions. Root pruning should be performed annually 1–4 weeks before flowering, on both sides of tree row, 30–40 cm from trunk, at depth of 25–30 cm. To improve regeneration of roots after pruning, soil moisture should be high. Therefore, root pruning gives particularly good results in irrigated apple orchards or in regions with high rainfalls during the spring and the summer when regeneration of injured roots is occurs.

As it was mentioned above, seed number affects fruit Ca. A high seed number encourages accumulation of Ca into fruit. It appears that seeds help direct the flow of Ca into fruit. Even though fruit with high seed numbers tend to be larger, the effect of Ca exceeds the size effect on fruit Ca so that these fruit with more seeds are both larger and richer in Ca. In bearing-orchards that do not have sufficient pollinator trees, it is necessary to plant apple trees of a suitable variety or topwork some trees or limbs with a variety that will provide the necessary pollen. It may be desirable to use honeybees to assist in cross-pollination.

10.3. Preharvest calcium sprays

Calcium sprays are one of the most effective treatment increasing fruit Ca. However, the efficiency of Ca sprays depends on the following factors:

10.3.1. *Calcium spray time*

Young fruit have usually higher ability to take up exogenous Ca per unit surface area than older ones (Michalczuk and Kubik, 1984). However, in some situations, uptake rate of exogenous Ca by mature fruit can be higher compared to young fruitlets (Glenn et al., 1985). This is due to the fact that during fruit growth, cracks and other surface irregularities may be formed which increases penetration rate of Ca into flesh tissues (Meyer, 1944; Wojcik et al., 1997). It is worth noting that Ca taken up by young fruit moves deeper into flesh tissues than Ca applied on surface of mature fruit. Thus, to reduce Ca-related physiological disorders occurring inside fruit such as internal breakdown and watercore, Ca sprays should be started just 2–3 weeks after petal fall. When fruit are affected by bitter pit or lenticel breakdown that symptoms occur on fruit skin and/or in outer cortex tissues, late season Ca sprays are more effective in reducing these disorders.

10.3.2. *Rate of calcium fertilizer*

Rates of application for most foliar Ca fertilizers range from 3 to 10 L/kg per ha. High Ca fertilizer rates increase generally Ca uptake rate by fruit (Wojcik, 1998b). However, at higher rates, injuries of leaf and fruit tissues occur. Young developing

leaves are particularly sensitive to injuries. Therefore, rates of the most Ca fertilizers, especially Ca salts without additives (Ca chloride or Ca nitrate) should be lower in early season by 20–30% compared to those applied in the fall.

10.3.3. *Spray technique*

Apple trees on vigorous rootstocks at high spacing (6×4 m, 5×4 m) require 1000–1500 L of water per ha to cover sufficiently total surface of leaves and fruit. Such high spray volumes are necessary due to the large volumes of tree canopy (Wojcik, 1998b). As planting density increases, tree size decreases which consequently leads to reduction of spray rates. Thus, in high density orchards, apple growers should use generally sprayers with low spray efficiencies. Required spray rate [R] in orchards can be calculated according to the formula:

$$R \text{ (L ha}^{-1}) = [H \times W_t/W_i] \times 330,$$

where H is tree height [m], W_t is tree canopy width [m] and W_i is interrow width [m]. Thus, higher spray rates are required when trees are high, tree canopy is wide and distance between tree rows is large. At spray rates below 300 L ha^{-1} it is necessary to lower the standard rate of Ca fertilizer by 10–20% since highly concentrated Ca solutions (>4%) can injury leaf and fruit tissues. When concentrated Ca material solutions (1.5–3.9%) are applied, sprays should be performed in the evening or at night since drying of solution from fruit surface is slow. In this way, surface-applied Ca have better conditions to move into fruit flesh tissues. Sprays of concentrated Ca solutions give good results in increasing fruit Ca if wind velocity is below 3 m·s^{-1} and tree canopy is loose. When wind velocity is higher and canopy is too dense, it is difficult to obtain uniformity of distribution of Ca solution within tree canopy. Additionally, in strong winds, drying out of Ca solution from fruit surface is quick which finally reduces rate of exogenous Ca uptake.

10.3.4. *Spray frequency*

Generally, with increasing number of Ca sprays during the growing season, fruit Ca status increases; although this relationship is not closely proportional (Grande et al., 1998). Under Polish conditions, from 3 to 8 sprays of Ca per season is recommended. Spray number is dependent on apple variety, the growing season and period of fruit storage. Apple varieties such as 'Jonagold', 'Szampion' 'Cortland' and 'Gloster' that are sensitive to Ca-related disorders, should be more frequently sprayed with Ca materials compared to varieties such as 'Lobo', 'Idared' and 'Elstar' rich usually in Ca. In dry seasons, number of Ca sprays in apple orchards should be high since under water stress conditions, accumulation of Ca into fruit is limited. Moreover, at high air temperatures, uptake rates of exogenous Ca by fruit are lower compared to those at moderate temperatures. Therefore, in hot seasons despite intensive Ca sprays it is difficult to obtain fruit rich in Ca. If it is predicted that fruit will be stored for long period of timing or will export on large distances, number

of Ca sprays in the growing season should be high. It is worth noting that too high number of Ca sprays results in decrease of fruit size and worsening their taste. Negative effect of intensive Ca spraying on apple quality is caused by reduction in photosynthesis rate resulting from decreased stomatal and mesophyll conductances (Swietlik et al., 1984).

10.3.5. *Calcium fertilizer quality*

In the Polish market, there are numerous commercial Ca-containing formulations. However, they usually contain less Ca compared to Ca chloride and Ca nitrate. Many of these commercial Ca materials have been studied to evaluate their efficiency in increasing fruit Ca. Generally, we have found that many of these Ca materials were less effective, and none of them were more effective than Ca chloride or Ca nitrate. Based on our experiments we can also suggest that the efficiency of Ca sprays in increasing this element in fruit depends mainly on amount of Ca applied. Thus, commercial materials rich in Ca should be generally more effective compared to those with lower Ca status.

Calcium chloride and Ca nitrate are often used foliar fertilizers to increase fruit Ca status. It is commonly believed that sprays with Ca chloride often result in leaf damage such as browning and death of the leaf margins (Raese and Drake, 1993). However, in many experiments we have observed no leaf injuries as a result of sprays with Ca chloride at rates from 3 to 8 kg·ha^{-1}. Problem with sprays with Ca chloride is potential corrosion of equipment. Therefore, it is imperative that equipment be cleaned thoroughly after spray with Ca chloride. In some growing seasons, sprays with Ca nitrate may deteriorate fruit colour. This is found particularly in years with high air temperatures occurring during 3–4 weeks before harvest. Under these conditions, development of the blush on fruit skin is reduced. Therefore, sprays with Ca nitrate should not be applied before harvest when weather conditions do not favour forming the blush on fruit surface.

10.3.6. *Weather conditions*

Temperature, humidity and air velocity have significant effects on the efficiency of Ca sprays. As fruit surface is long wetted with spray solution, Ca uptake by fruit increases. At high temperatures and low humidity of air, drying of Ca solution from fruit surface increases which finally reduces rate of Ca absorption. Therefore, fruit from the canopy top exposed to high temperatures have generally lower ability to absorb exogenous Ca compared to those from the bottom and the inside canopy. In strong winds during spraying, deposit of Ca solution within canopy is unevenly. Moreover, when it is windy, spray solution from fruit surface dries quickly which finally reduces Ca uptake by fruit.

Taking into consideration profits resulting from production of fruit rich in Ca and the high efficiency of surface-applied Ca uptake, we claim that Ca sprays should be routine treatment yearly, applied particularly on apple varieties sensitive to Ca-related physiological disorders.

10.4. Postharvest calcium treatments

In many cases, orchard treatments are not successful to produce fruit with adequate Ca levels. Therefore, in many countries postharvest Ca treatments are recommended (Conway et al., 1994). It is commonly expected that postharvest Ca application is more effective in reducing Ca-related disorders than orchard Ca sprays. However, we think that postharvest Ca treatment should be viewed as a supplement to pre-harvest Ca application.

   Three modes of postharvest Ca treatment can be distinguished: dip or drench, vacuum infiltration and pressure infiltration (Fallahi, 1997). Both vacuum and pressure infiltrations have been used commercially to some extent and found to be unsatisfactory (Hewitt and Watkins, 1991). Therefore, we will focus below on the efficiency of fruit dipping in Ca solution. This treatment usually eliminates and/or reduces the incidence of bitter pit and senescent breakdown during and following fruit storage. Sometimes, this treatment can reduce scald development and rotting and maintain fruit firmness as well. However, we claim that postharvest Ca treatment should be viewed as a method to protect and/or reduce fruit against development of bitter pit and senescent breakdown. Other benefits resulting from postharvest Ca treatment occur occasionally.

   In postharvest treatment, only Ca chloride is used but its use is limited to purities of 94 percent or greater. Fruit are dipped usually in 1.5–2.0% solutions of Ca chloride which is sufficient to control bitter pit and senescent breakdown (Roy et al., 1994). If it is needed to improve firmness and rot control, fruit should be dipped in 4% Ca chloride solution. However at such high concentration of Ca chloride solutions, the risk of fruit surface injury increases. The efficiency of fruit dipping depends not only on Ca concentration in solution but also on temperatures of fruit and solution, time of fruit dipping, humidity of storage atmosphere and on applied additives into solution. If warm fruit are put into cold Ca solution, air inside the fruit contracts as it cools. This creates a small vacuum inside the fruit and solution is sucked into the apple (Lee and Dewey, 1981). If cold fruit are put into warm water, air in the fruit warms and expands, creating pressure that blocks entry of solution into the fruit. Therefore, solution temperature should be about the same as fruit temperature for consistent responses. To cover fruit surface with Ca solution, fruit should be dipped for 20–30 seconds. For a drench treatment, about 1 minute is required for complete fruit surface wetting. Too short time of fruit dipping gives worse results. Because postharvest Ca treatments may cause fruit surface injury, fruit after dipping are often washed to remove the excess of Ca chloride. Fruit washing is done about 5–7 days after treatment. Washing shortly after fruit dipping dramatically reduces Ca uptake. High humidity of storage atmosphere increases the efficiency of treatment because Ca is taken up from residue only as long as the residue is in a liquid form. If residue dries out, Ca uptake by fruit ceases. When relative humidity is higher than 90%, the residue does not dry out and Ca absorption proceeds throughout the storage period. Therefore, fruit postharvest Ca treated should not be stored at relative humidity below 90%. Additives to the dip solution may also increase the efficiency postharvest Ca treatment by getting more thorough surface coverage with residue, increasing the amount of Ca bound to the

surface, or by increasing Ca penetration in to the fruit. Surfactants such as lecithin or opron oil increase Ca coverage on fruit surface and/or improve Ca penetration through surface openings in to the fruit (Mason et al., 1974). To increase the efficiency of postharvest Ca treatment, also thickeners such as ketrol and cornflour are added in Ca solution (Johnson, 1979). However, thickeners with Ca chloride may create a visible residue on the fruit surface that are difficult to remove by washing.

The great risk with postharvest Ca treatment is related to fruit skin injury (Conway et al., 1994). There are two forms of injuries: lenticel spotting and calyx bronzing. Lenticel spotting is difficult to detect during fruit storage. However, after removal of fruit from storage, symptoms of lenticel spotting creates as small sunken areas around the lenticels. Latter, these spots turn greater and black and if they are abundant, they become clearly evident. Calyx bronzing is usually evident at removal of fruit from storage but rarely deteriorates external fruit quality, at least on red apples. It should be noted that lenticel spotting or calyx bronzing is not only related to postharvest Ca treatment because symptoms of these damages are also observed on fruit untreated with Ca. Thus, postharvest Ca application stimulates rather than induces the occurrence of these injuries on fruit skin.

11. CONCLUSIONS

Without doubt, Ca plays a critical role in apple quality. Fruit with low Ca concentrations have generally low storage potential which causes a great losses to growers. Deficiency of Ca in fruit flesh is common phenomenon. Therefore, it is important that cultural practices in apple orchards promote accumulation of Ca into fruit tissues. The following factors stimulate Ca transport into fruit: soil pH 5.5 to 6.5, balanced fertilization, particularly with N, K and Mg, application of gypsum and $N-NO_3$ fertilizers to soil, moderate moisture and adequate soil structure, controlled thinning of flowers or fruitlets, moderate dormant pruning, and application of summer pruning and root pruning. Despite significant effects of the above-mentioned factors on fruit Ca status, Ca sprays during the growing seasons are necessary, particularly on apple cultivars sensitive to Ca-related physiological disorders. However, the efficiency of Ca sprays depends on many factors such as treatment time, quality and rate of Ca fertilizer, spray number, spray technique and weather conditions during and after treatment. Postharvest fruit dipping in Ca solution is usually more effective in increasing fruit Ca than sprays of Ca in orchards. Postharvest Ca treatment is particularly beneficial in reducing bitter pit and senescent breakdown development. However, this treatment should be viewed as a supplement to preharvest Ca sprays.

It should be noted that high storage potential of fruit is related not only to Ca level but also to the efficiency of protection against pathogens, harvest date, velocity of fruit cooling after harvest, and storage conditions.

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DIAGNOSIS, PREDICTION AND CONTROL OF BORON DEFICIENCY IN OLIVE TREES

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1. INTRODUCTION

Boron (B) is an essential micronutrient to plant growth as it was shown by Warington (1923). Lack or deficiency of B results in rapid inhibition of plant growth that is attributed to the specific structural role that B plays in the cell wall and the limited mobility of B in most of the plants (Hu and Brown, 1997). Boron deficiency in olive trees was first observed by Horne (1917) as an unusual disease called 'exanthema' that was characterized by withering of tips of shoots resulting in bushy growth die back of branches and puffed bark. The disease was proved that is caused by lack of B after disappearing of the above mentioned symptoms by treating the branches with B (Scott et al., 1943).

Boron deficiency in crops is more widespread than deficiency of any other micronutrient. This is the main reason why numerous reports were published on B-deficiency in plants. Boron lack was referred in many parts in all over the world such as Asia, Europe, Africa, Australia, South America, and the USA (Shorrocks, 1997). Olive (*Olea Europea*, L.) is considered a susceptible to B deficiency crop (Shorrocks, 1989). Among the countries in which olive is cultivated, B deficiency was recorded in many of them like USA (California), Italy, Greece, and Israel (Hartman et al., 1966; Tsadilas and Chartzoulakis, 1999). Boron deficiency may seriously affect olive's yield.

2. DIAGNOSIS OF BORON DEFICIENCY IN OLIVES

Diagnosis is the determination of nutrient status in a plant at the time of sampling (Smith, 1986). For diagnosis usually both the existence of macroscopic plant symptoms as well as plant analysis data are utilized.

2.1. Plant symptoms of boron deficiency in olives

Boron deficiency symptoms in olives are quite distinctive. They can be seen on all the parts of the trees i.e. leaves, branches, and fruits. The most typical symptom of a severely affected olive tree is the plethora of dry branches distributed in the whole tree. The terminal buds of the shoots usually die and the growth continues with shoots from developing lateral buds, that again die, replaced by new lateral shoots and so on. The final result is the formation of 'witches broom' type of growth. The terminal bud death usually happens in the midsummer while at the same

Figure 1. Severely affected by B deficiency olive tree (A) and pronounced evolution of boron deficiency symptoms in olive leaves, malformed boron deficient olive leaves and dead buds in a young branch (B) (Figure C. Tsadilas).

season two consecutive bud growth and death may happen. A view of a severely affected olive tree by B deficiency is shown in Figure 1A.

Boron deficiency symptoms in olive tree leaves start to appear in the new shoots at the beginning of July. They are characterized by a pale green color in the tips reaching about one to two thirds of the leaf. Since the olive trees are evergreen these symptoms may be observed for several seasons at all times. In severe B deficient trees the leaf tips become yellow or orange and finally necrotic, leaving a yellow-banded region between the tip and the base of the leaf. The final result is a considerable leaf dropping and the foliage becomes sparse. The leaves frequently are deformed and curl. A pronounced evolution of the B deficiency symptoms in olive leaves is clearly shown in Figure 1B.

In some cases of severely affected by B deficiency olive trees the bark of larger branches may produce protuberances 2 mm high and 5 to 10 mm long underneath of which there is brown necrotic tissue (Hartman et al., 1966). Usually the bark cracks and becomes roughened.

Boron deficiency substantially affects olive trees yield. In severely B deficient trees flower-bearing eyes are not formed and inflorescence are not developed in the spring. Olive trees with mild to moderate B deficiency symptoms in leaves appear to blossom and set fruit normally. However later in July and August most of the immature fruits may drop. Fruits of B deficient trees are usually malformed or defective. They have pits, they are shrivelling and drying in the apical half or less like forming a characteristic appearance usually called 'monkey face' (Scott et al., 1943). These parts of the fruits finally become necrotic.

2.2. Plant analysis of boron deficient trees

In order tissue analysis to provide a base for a quantitative assessment of nutrient status of a plant, a good understanding of the physiology of the element under

consideration is required. The role of B in plant physiology is not completely known yet. Therefore, B tissue analysis serves only as an empirical tool in B nutrition assessment. It is based on standards that have been established to distinguish adequate from deficient B concentrations. The establishment of standards requires good knowledge of the factors that may affect the interpretation. Such factors are plant parts sampled, plant age, plant species and cultivar and environmental conditions (Bell, 1997). For olive, as well as for several other plants, plant parts to be analysed for diagnosis purposes are leaves. However, the plant parts from which samples must be taken and the sampling period for olive trees are questionable. In general, it is acceptable that in species that cannot retranslocate B, young growing plant parts are preferable than recently matured parts for B deficiency diagnosis. This is true especially for phloem immobile or variably mobile elements (Bell, 1997). Boron is a low phloem mobile element (Marschner, 1986) as it was supported by several observations indicating that a lack of B is usually found in young developing tissues (Eaton, 1944). Oerti and Richardson (1970) have also suggested that B does not readily move out of mature leaves. However, there are a few studies that suggest that B can move from mature leaves to other plant parts (Hanson, 1991). Delgado et al., 1994) in a recent study concluded that B may be mobilized from young leaves during anthesis to supply B requirements of flowers and young fruits. Taking into account all the above-mentioned, it is concluded that plant parts for B diagnosis purposes should be young growing parts.

However, most of the researchers (Brito, 1971; Chaves et al., 1967; Recalde and Esteban, 1968; Samish et al., 1961; Bouat, 1968) agree that sampling in olives must be done during the winter dormancy by collecting the leaves from the middle of one year branches, i.e leaves 5–8 months old. In this period the chemical composition of the leaves is relatively constant while during the growing period it changes rapidly in short periods. Jones et al. (1991) suggest that for diagnosis of B deficiency in olives 50 fully expanded leaves from midshoot, in non specified time, must be taken.

Several researchers have worked on finding standards of B nutritional status for olive (Table 1). Hansen (1945) found B concentration in olive leaves from severely B deficient trees 7–13 μg/g dry matter. In trees with slight or no B deficiency symptoms, but responded to B application, B concentration was 14–15 μg/g. Boron concentration ranging between 16–18 μg/g was doubtful while B concentration above 19 μg/g was adequate for normal plant growth. In the same study B concentration in fruits showing B deficiency symptoms ranged between 3.4–3.5 μg/g while in the healthy fruits it was above 20 μg/g. Similar results were also reported by others. Demetriades et al. (1960) reported that B deficiency appears in olive tree leaves with B concentration below 14.5 μg/g in various areas of Greece. With the results of these authors agree the findings of other researchers in Greece (Tsadilas et al., 1994a; Tsadilas et al., 1994b; Tsadilas, 1995; Tsadilas and Chartzoulakis, 1999). In general, it can be concluded that slight B deficiency symptoms begin in the olive trees when B concentration in leaves ranges between 15–20 μg/g while in B concentration below 15 μg/g the symptoms become apparent and distinct (Tsadilas, 1995).

Table 1. Boron nutritional status of olive trees with respect to leaf boron concentration.

Deficiency	Low	Normal	Excess	Reference
7–13	14–18	19–33	268	Hansen, 1945
<15	–	16–30	196	Demetriades et al., 1960; Demetriades and Holevas, 1968;
<15	15–20	20–180	>250	Shorrocks, 1989
<15	15–20	>20	–	Tsadilas, 1995; Tsadilas et al., 1994a; Tsadilas et al., 1994b; Tsadilas and Chartzoulakis, 1999
–	15–19	20–75	>75	Jones et al., 1991

2.3. Field response to boron fertilizers

The final proof that the nutritional disorder under consideration was due to lack of B, is the correction of B deficiency symptoms after addition of B fertilizers to the trees. This requires field experiments that are very difficult because several factors are involved and affect plant growth. So, in order the experiment to be successful, B lack must be the only or the most significant limiting factor in olive growth. Boron in the experiments can be given either by foliar and soil application.

3. PROGNOSIS OF BORON DEFICIENCY IN OLIVES

Prognosis is the prediction of the possibility of a B deficiency that may impair plant growth at a later stage in the growth cycle after the sample was taken (Smith, 1986). This approach uses both soil and plant analysis. The basic difference of prognosis from diagnosis is on the time that elapses between the sample collection and the measurement of the effect on the later plant growth. In soil analysis the time interval is the entire growth period while in the case of plant analysis the time interval is shorter (Bell, 1997).

3.1. Plant analysis for prognosis of boron deficiency in olives

In order plant analysis to serve prognosis of B deficiency, plant parts that reflect nutrient supply from the soil or nutrient reserves within the plant should be selected. Unfortunately, there are only a very few studies referred to the plant parts that can be used to assess their efficacy in predicting yield. Generally, in plants that retranslocate B the water-soluble B fraction may be a good predictor of the possibility of a subsequent B deficiency (Bell, 1997). Since the relevant research data are very few, usually the same plant parts used for diagnostic standards are also used for prognosis of B deficiency. This can lead to wrong estimations as it was proved for potato by Pregno and Armour (1992). Similar weakness there is also for issues related to plant age or environmental factors involved in prognosis of B deficiency

in olives. Olive is a plant that cannot retranslocate B easily (Gavalas, 1978), so plant analysis alone for B deficiency prognosis is yet doubtful.

3.2. Soil analysis for prognosis

While plant analysis alone is not a safe procedure for predicting B deficiency in olive, as it was discussed above, plant analysis in combination with soil analysis could be more effective. The respective approach could include the formation of functions between soil and leaf analyses data, which could be used for establishing threshold values for soil analyses below which B deficiency is expected to develop. This approach introduces the necessity of studying the efficacy of methods determining available soil B. Such efforts trying to find relationships between available soil B forms and B concentration in leaves usually fail for tree crops. The main reason for this is that the sampled soil volume is difficult to be representative of these crops that develop a very deep root system. However, there are some encouraging data on this topic that will be discussed later.

The most common method used for assessing available soil B is the hot water (or 0.01 M CaCl$_2$) extraction introduced by Berger and Truog (1939). For olive, the optimum value of hot water extractable B was proposed to be the range 0.1–0.5 µgB kg^{-1} soil (Berger, 1949). However, in many cases this method is not a good predictor of available soil B as well as it is time consuming and requires special B free glassware. To overcome all these difficulties several alternatives to this method were proposed (Gupta et al., 1985). Among these the following methods are included: saturation extract (and conversion of it to soil solution B, Gupta, 1968), extraction with dilute HCl solution (Ponnamperuma et al. (1981), extraction with 0.01 M mannitol–0.01 M CaCl$_2$ solution (Cartwright et al., 1983), ammonium acetate and sodium bicarbonate extraction (Schuppli, 1986), hydroxylamine HCl, and ammonium oxalate extraction (Tsadilas et al., 1994a; Tsadilas and Chartzoulakis, 1999). For all the above mentioned methods attempts were made to correlate soil B concentration with that in dry matter of the plants with variant success. Tsadilas et al. (1994a) and Tsadilas and Chartzoulakis (1999) tested many of these procedures for olive and estimated the threshold values for B deficiency. In the relevant regression equations they put as dependent variable the concentration of soil B and as independent the standard value of B concentration in dry matter in olive leaves that correspond to B deficiency. A summary of their results is shown in Table 2. However, it is obvious that inclusion into these equations of some other factors affecting B uptake by plants, such as soil water, soil texture, soil pH, and sesquioxides content clearly improve the correlation coefficients of the respective equations. For example, Tsadilas et al. (1994b) found that inclusion of amorphous iron oxides concentration in the regression equation with dependent variable the hot water soluble B and independent variable the concentration of B in olive leaves, significantly increased the correlation coefficient.

The failure to predict B deficiency using single critical B concentrations, besides to the above-mentioned factors, may also be attributed to the fact that the various extractants extract B from different pools retaining B with different strength. That

Table 2. Critical values of soil B extracted by different extractants for appearance B deficiency in olive (Tsadilas et al., 1994b).

Extraction procedure	Critical value, $\mu g\ kg^{-1}$ soil
Hot water	<0.33
Cold water	0.17
0.01 *M* HCl	<0.05
0.05 *M* mannitol in 0.01 *M* CaCl$_2$	<0.41
Hydroxylamine HCl	<0.14

is the reason that attempts started to be made to fractionate B into fractions and find their availability to the plants. In such an attempt, Tsadilas et al. (1994c) fractionated B into soil solution B, non specifically adsorbed B, specifically adsorbed B, B occluded in Mn oxyhydroxides, and B associated with silicate minerals. From these fractions available to olive were found to be soil solution B, specifically adsorbed B, B occluded in Mn oxyhydroxides, and in amorphous iron oxides. Much more such studies are needed, in order to find good indicators for available soil B to the plants.

4. BORON DEFICIENCY CURE IN OLIVE

Boron cure in B deficient olive trees is easy and inexpensive. Borates can be applied either to the soil or sprayed on to the foliage. Application to the soil must ensure uniform distribution of borates since the amount of borates required is very small. From the other hand it is well known that the range between the concentration in soil that causes B deficiency and the one that causes B toxicity, is very narrow. So, if the borates are not uniformly applied, it is very possible, in some areas of the field, B toxicity to be caused while other parts may left in B deficiency state. Boron compounds, mainly used as fertilizers, are shown in Table 3.

Borates can be applied in solid or solution form alone or together with fertilizers or pesticides with which are compatible. Foliar application is preferred in cases that no enough rainfall is expected. For this case solubor is considered the ideal material (Shorrocks, 1989) since it is very soluble in water having high solubility and it is compatible with several insecticides, fungicides or herbicides.

Table 3. Boron compound used as B fertilizers (Shorrocks, 1989).

Material	% B	Amount of material required (kg) for 1 kg B
Fertilizer borate 47	14.8	6.76
Fertilizer borate 48	14.9	6.71
Borax	11.3	8.85
Boric acid	17.5	5.71
Solubor	20.8	4.81

The normal concentration of solubor is 0.2–0.5% w/v. To ensure a good supply of B throughout the growing season it is usual to split the total rate into two or more applications.

However, the most practical way of B application is to apply that as borax or boric acid to the soil. The recommended rates for olive vary widely. Hansen (1945) treated B deficient olive trees in California by spraying them with borax or applying borax in the soil in rates 220 to 450 g per tree achieving to care the disorder. A rate of about 450 g per tree was considered adequate for complete care. Similar results were obtained by Demetriades et al. (1960) in some trials carried out in the island Lesvos, Greece. Shorrocks (1989) suggested a rate of 1–3 kg B/ha.

Tsadilas et al. (1994b), in order to study the problem more systematically by using apart from plant analysis also soil analysis data, carried out experiment similar to that of Demetriades et al. (1960). The experiment was established in 1991 in an area of Larissa with B deficient olive trees var. 'Amfissa', which is one of the best table varieties in Greece. The experimental design was latin square with 4 treatments: 0, 200, 350, and 500 g borax per tree, each replicated four times. Borax was incorporated in the soil (5–10 cm depth) in a band around the trunk of the trees in a distance about 30 cm away from them very early in the spring. The soil was a Typic Xerorthent, shallow (50 cm deep), with a slope 8 to 10%, sandy loamy, acid (pH 5.5) but relatively rich in organic matter (2.5–3.00%) due to the manure that is traditionally used in the area. The trees were sufficiently irrigated during the whole growth period. Next August leaf samples were collected from all the trees of the experiment from the current vegetation (well developed leaves from the middle of new branches) and analyzed for B. Composite soil samples were also selected and analyzed for available soil B. The results are shown in the Table 4. From the data of Table 4 it is clear that B deficient olive trees significantly responded to borax application. The symptoms of B deficiency in the new branches were disappeared (Figure 2C, 2D). Borax rates of 200 g/tree, are considered adequate for the conditions of this experiment for curing B deficiency problem in olive trees.

Table 4. Influence of B fertilization on olive leaf and available soil B concentration (Tsadilas et al., 1994b).

Borax applied g/tree	B concentration in olive leaves μg/g d.m.	Available soil B concentration μg/g soil
0	9.4c*	0.32b
200	46.7b	7.52a
350	53.0b	9.14a
500	80.9a	9.16a

* Different letters in the same column denotes statistically significant diefferences at the probability level.
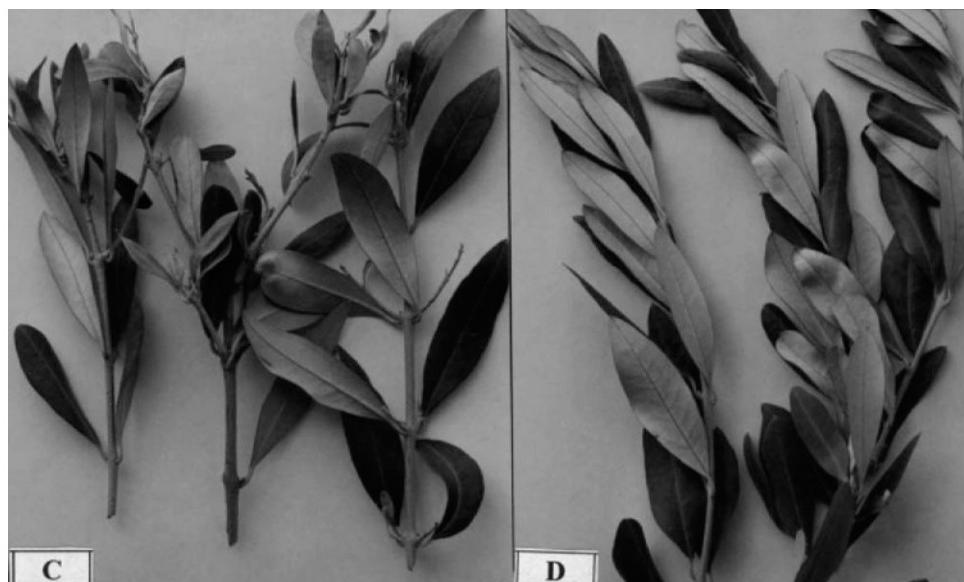
Figure 2. Branches from a boron deficient olive tree before (C) and after boron application (B) (Figure C. Tsadilas)

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BORON-CALCIUM RELATIONSHIP IN BIOLOGICAL NITROGEN FIXATION UNDER PHYSIOLOGICAL AND SALT-STRESSING CONDITIONS

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1. BORON IN LIVING ORGANISMS

The boron atom is small and has only three valence electrons and intermediate properties between metals and non-metals. These features lead to a unique chemistry. With the possible exception of carbon, B has the most interesting and diverse chemistry of any element (Power and Woods, 1997). Despite of its importance, B, along with its neighbours Li and Be, are not abundant elements, as they are bypassed in the normal chain of thermonuclear reactions in stars. Recent works have proposed that B might also be produced during explosions of massive stars. In spite of its low natural abundance, B is widely distributed both in the lithosphere and the hydrosphere. Usually, only soluble B (about 10% of total B in soil) is available to plants. Moreover, boron deficiency is more common than deficiency in any other plant micronutrient, worldwide. On the other hand, the special interest for biochemists and physiologists on the role of boron is because it is the only known element to be required for higher plants, without a role in animals, algae (except diatoms), and fungi.

It is now more than 70 years since boron was convincingly demonstrated to be essential for normal growth of higher plants. However, the biochemical role of B is not well understood at the moment, and unlike other micronutrients, it has not been shown to be a component of any enzyme system. Boron deficiency causes many anatomical, physiological, and biochemical changes, most of which represent indirect effects. Because of the rapid onset and the wide variety of symptoms following B deprivation, determining its primary function in plants has been one of the greatest challenges in plant physiology. Several recent reviews propose that B is implicated in three main processes: keeping cell wall structure, maintaining membrane function, and supporting metabolic activities. However, in the absence of conclusive evidence, the primary role of boron in plants remains elusive (Blevins and Lukaszewski, 1998). Recent research has confirmed *in vivo* the proposed role of B for cell wall architecture (O'Neill et al., 2001) and has opened new research topics such as bacterial quorum sensing (Chen et al., 2002; Coulthurst et al., 2002). The essentiality of boron in both cases is based in the well-known capability of boric acid/borate mixtures to form complexes with sugars or other compounds with cis-hydroxyl groups.

The diversity of roles played by B might indicate that either the micronutrient is involved in numerous processes or that its deficiency has a pleiotropic effect. Based on data from literature, it is very likely that the main reason for B essen-

tiality is the stabilisation of molecules with cis-diol groups turning them effective, irrespectively of their function. It is possible that new roles for B, based on its special chemistry would appear. The recent reports on the requirement of boron for cross-linking of the cell wall rhamnogalacturonan II component (O'Neill et al., 2001) as ligand in the cyclic furanosyl diester bacterial quorum-sensing signal AI-2 (Chen et al., 2002), and for vesicle targeting and transmembrane transport in symbio-somes (Bolaños et al., 2001), evidence a role of B as a 'molecular staple'.

It is very difficult to find a better candidate for atomic diester bridging in these complex molecules. Although other atoms, such as phosphorous or sulphur, might make links through diester bridges, the resultant configuration would be highly unstable due to the electron density of those heavier atoms, whereas the simpler borate-diester is very stable.

2. ROLE OF BORON IN FREE-LIVING NITROGEN-FIXING MICROORGANISMS

2.1. Boron in cyanobacteria

Among dinitrogen fixing microorganisms, Cyanobacteria, or blue-green algae, form a remarkable group because they have oxygenic photosynthesis that probably made cyanobacteria responsible for the major evolutionary transformation of the biosphere, leading to the development of aerobic metabolism on Earth three billion years ago. In evolutionary terms, they represent a link between bacteria and green plants. Their cellular organisation, known as prokaryotic is characterised by a lack of membrane bound organelles, however, their principal mode of nutrition, oxygen evolving photosynthesis is similar to that which operates in all other nucleate algae and higher plants. Therefore, the cyanobacteria provide a biologically simple model for studying problems in mineral nutrition, especially of boron and calcium not only in relation to nitrogen fixation but also to photosynthesis and typical plant processes.

The requirement of boron is not a general feature in cyanobacteria. For example, evidence is presented that boron is not required for the growth of *Anacystis nidulans* (*Synechococcus* PCC7942) (Martínez et al., 1986). Furthermore, *Anabaena* PCC7119 a dinitrogen-fixing cyanobacterium, growing in the presence of combined nitrogen was not affected by boron deficiency.

However, when this microorganism was grown under dinitrogen-fixing conditions lacking any boron, inhibition of growth and deficiency of photosynthetic pigment proteins were observed (Mateo et al., 1986).

Inhibition of growth resulting from boron deficiency was reversible by B addition or by supply of combined nitrogen. These findings are consistent with the pre-ceding data and suggest that boron is only required by *Anabaena* when cells are growing under dinitrogen-fixing conditions.

The study of dinitrogen fixation by the acetylene reduction method indicated that nitrogenase activity of boron deficient cells was reduced to about 40% of those activities observed in the boron supplied cells within the first two hours of

Table 1. Effects of boron deficiency on dry weight (mg mL^{-1}), protein (μg^{g-1} dw) and pigment contents (μg^{g-1} dw) of *Anabaena* PCC7119 after 96 h of growth in media containing NO_3^- or under nitrogen fixing conditions (N_2).

	Dry weight	Protein	Chlorophyll	Phycobiliproteins
NO_3^- +B	1.20 ± 0.20	496 ± 31	11.9 ± 3.0	97.3 ± 16.5
NO_3^- –B	1.12 ± 0.20	482 ± 28	11.4 ± 2.0	93.8 ± 18.0
N_2 +B	0.40 ± 0.10	437 ± 32	11.3 ± 2.0	115.4 ± 22.0
N_2 –B	0.16 ± 0.05	208 ± 15	6.5 ± 0.8	42.9 ± 7.0

Table 2. Effects of boron deficiency on nitrogenase activity (μmol C_2H_4 mg^{-1} chl h^{-1}) of *Anabaena* PCC 7119.

	1 h	% inhib.	2 h	% inhib.	3 h	% inhib.	24 h	% inhib.
+B	22.5 ± 2.5		57.1 ± 5.4		104.1 ± 10.8		836.0 ± 57.5	
–B	20.2 ± 2.4	10.3	32.8 ± 2.9	42.5	56.1 ± 4.8	46.1	7.2 ± 1.2	99.1

culture. There was no detectable nitrogenase activity in cultures after 24 h of boron deficiency. At this time other metabolic processes (such as photosynthesis) were not affected by boron deficiency. Therefore, boron may have a role in dinitrogen-fixation in cyanobacteria. However, nitrogenase synthesis was not affected by boron deficiency, indicating other role of B not directly related with the enzyme activity (García-González et al., 1988).

Nitrogen fixation is a anaerobic process and all nitrogenase components are rapidly destroyed by oxygen. Heterocysts are specialised cells present in some filamentous cyanobacteria when grow in the absence of a combined nitrogen source. These cells are capable of aerobically fix N_2 because they maintain the reducing environment required for cyanobacterial nitrogenase activity. There may be several complementary mechanisms that enhance the effectiveness of heterocysts as sites for dinitrogen fixation: the general reducing environment, the high activities of some enzymes of oxidative penthoses pathway (OPP), a lack of photosynthetic oxygen evolution, enhanced superoxide dismutase, etc. However, the most conspicuous of these mechanisms is the presence of a thick envelope in the heterocyst. This envelope is comprised of a inner laminated layer, a central homogeneous layer consists of specific glycolipids that are absent in vegetative cells (Lambein and Wolk, 1973). It has been suggest that these glycolipids provide a barrier to the diffusion of oxygen.

Examination of boron starved cultures clearly shows important changes in the morphology and ultra structure of the heterocysts (Figure 1) not only in *Anabaena* but also in other cyanobacteria (Bonilla et al., 1990). It was proposed that boron might be involved in stabilising the heterocyst envelope. Quantification by HPLC of glycolipids in heterocyst envelopes of B-deficient cultures showed that the amount of these components was less than 15% of that in the control after 24 hours of B deprivation (García-González et al., 1991). These results clearly show that boron
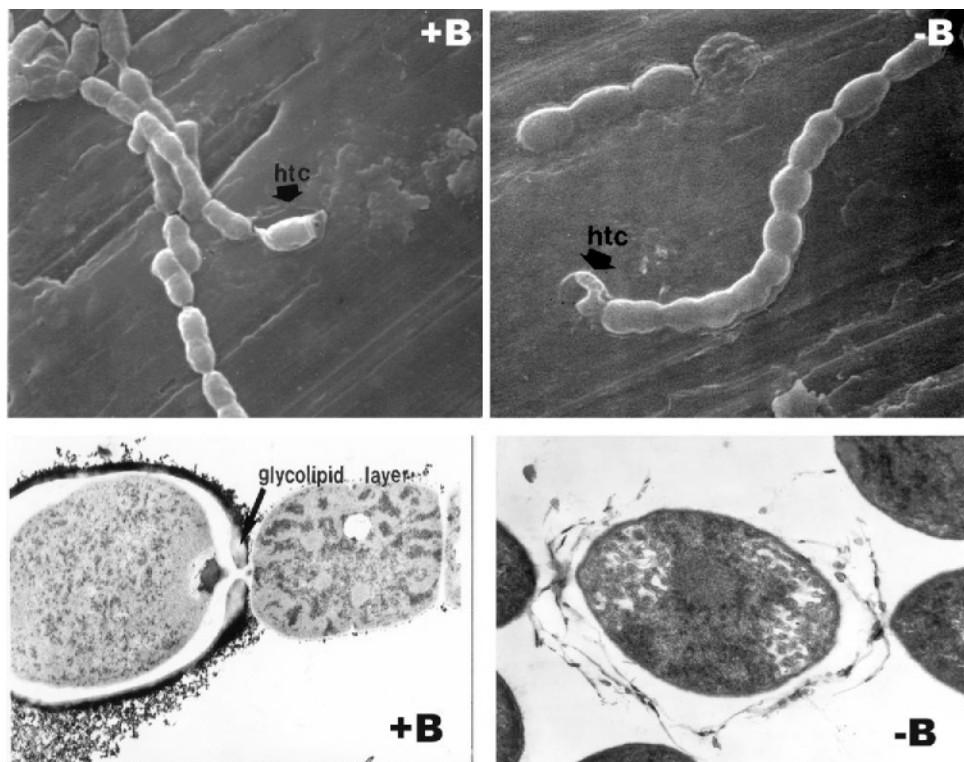
Figure 1. Heterocysts of *Anabaena* PCC 7119 grown in the presence (+B) or in the absence (–B) of boron. Scanning electron microscopy (upper side) shows collapsed heterocysts in B-deficient filaments. Degeneration of the envelope and the absence of the glycolipid layer show by transmission electron microscopy (bottom side) in –B indicates a role of the micronutrient in the stabilisation of these components.

is an essential element for stabilizing the inner glycolipid layer of the heterocyst envelope. Therefore, the result of B deficiency is the inhibition of nitrogenase activity by the massive oxygen entry inside the heterocyst.

In response to boron deficiency, there is also a short-term increase in the activities of the mechanisms that protect nitrogenase in heterocysts against oxygen inhibition: increases in SOD, catalase and peroxidase (García-González et al., 1988), as well as respiration, and the OPP (García-González et al., 1990).

2.2. Boron in *Frankia*

A nitrogen-fixing bacterium with structural and functional similarities to heterocystous cyanobacteria is the actinomycete *Frankia*. Bacteria of the genus *Frankia* form so-called actinorhizal symbioses with several non-leguminous shrubs and trees termed actinorhizal plants, wherein the endophytic form of the microsymbiont develops the N_2-fixing activity (Wall, 2000).

Similar to cyanobacteria, but different to rhizobia, *Frankia* strains isolated from

nodules can fix N_2 when cultured without a nitrogen-combined source. Nitrogenase in free-living cultures or in symbiotic state is localised inside the specialised vesicles that differentiate from some filament tips (Huss-Danell, 1997). The N_2-fixing vesicle is in many ways structurally and functionally analogous to the heterocyst (Zehr, 1998). Therefore, based on the similarity to heterocysts, B has been demonstrated to be essential not only for the development of the actinorhizal symbioses but also for the differentiation of N_2-fixing vesicles of *Frankia* as in heterocystous cyanobacteria (Bolaños et al., 2002a).

Frankia BCU110501, a strain isolated from *Discaria trinervis* nodules (Chaia, 1998) was unable to grow (Figure 2) in B deficient conditions. Filaments of B-deficient cultures are sorter than normal, and development of functional N_2-fixing vesicles is inhibited in the absence of the micronutrient (Figure 3).

The protection of nitrogenase activity against oxygen diffusion is attributed to the resistance properties of the lipidic multilaminate vesicle wall (Parsons et al., 1987), which can change its thickness by modifying the number or lipidic monolayers in response to different pO_2 (Harris and Silvester, 1992). The analysis of lipids showed that vesicles have a higher content of glycolipids and neutral lipids than vegetative cells, being the major proportion long-chain polyhydroxy fatty acids or alcohols
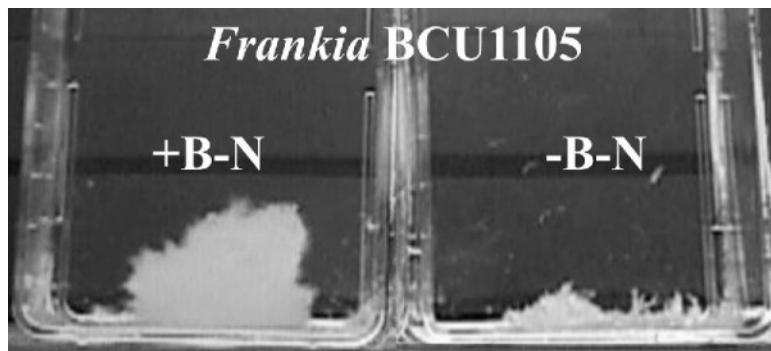

*Figure 2.* Liquid cultures of *Frankia* BCU110501 in media with (+B) or without (–B) boron.
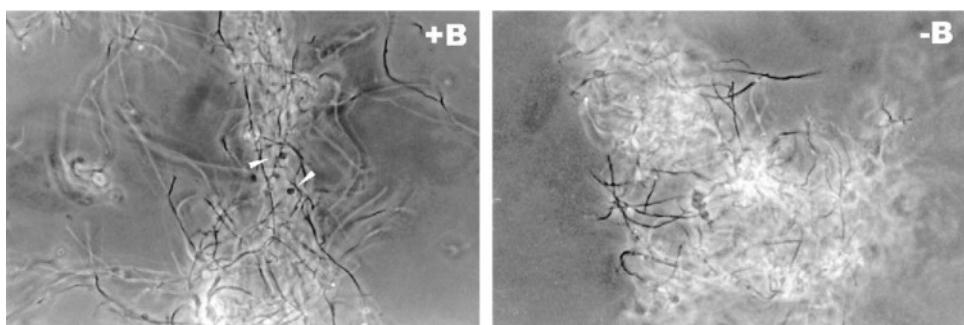
Figure 3. Filaments of *Frankia* BCU110501 developed in the presence (+B) or in the absence (–B) of boron. Boron deficiency leads to short filaments that do not develop N_2-fixing vesicles (highlighted by arrowheads in +B).

(Tunlid et al., 1989). A very high concentration of the hopanoid bacteriohopanetetrol is also present (Berry et al., 1991). All of these constituents of the vesicle envelope are compounds rich in diol groups which can interact with borate ions. The appearance of B-deficient vesicles is similar to that reported for B-starved heterocysts (García-González et al., 1991), which is due to the loss of the inner laminated layer of the heterocyst envelope. That layer is composed of glycolipids with long-chain polyhydroxyl alcohols (Lambein and Wolk, 1973) stabilized by boric acid. The place were the lipidic envelope is supposed to be (Torrey and Callaham, 1982) is very narrow inside B-deficient vesicles, suggesting also a thinner laminated envelope. Therefore, B can play a role in the stabilisation of vesicle envelope (Figure 4), as the micronutrient does in the heterocysts.

Contrary to cyanobacteria, the micronutrient is also needed for *Frankia* vegetative growth when the bacteria are cultivated in media containing combined nitrogen. Boron deficient filaments in either culture are thinner and with a twisted appearance that indicate an altered surface (Figure 5). The inner laminated layer of the heterocyst envelope stabilized by B is composed of lipids not found in vegetative cells (Nichols and Wood, 1968), while vesicle envelope is enriched in lipids that are also constitutive of filaments. This particular difference could explain why B is also needed for the structure and growth of vegetative cells of *Frankia* BCU110501.
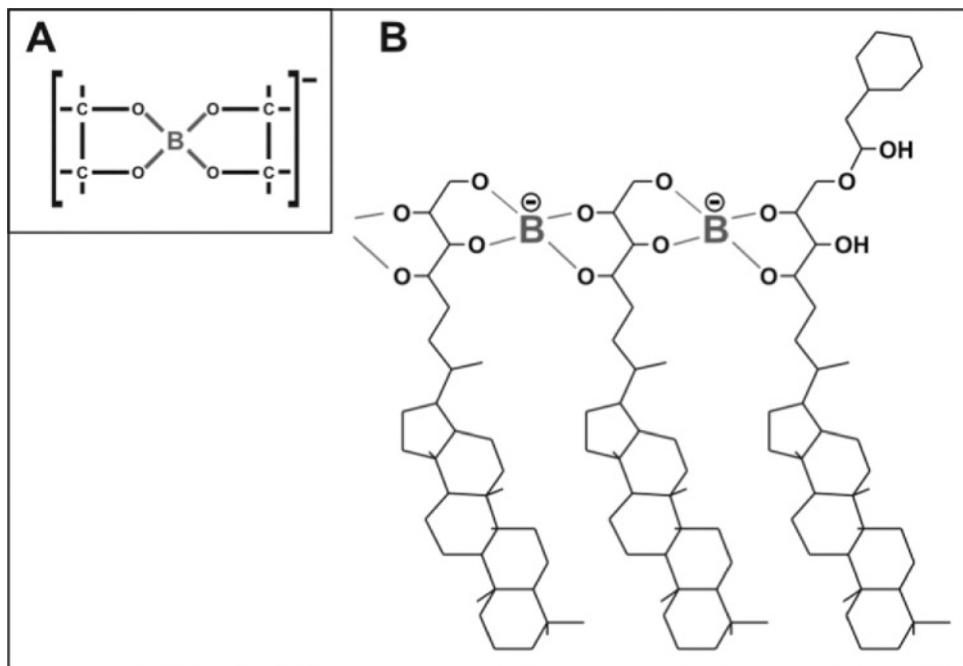

Figure 4. Bis(diol) borate complex (A). Hypothetical model for bacteriohopanetetrol and bacterio-hopanetetrol phenylacetate linkage by boron in the envelope of *Frankia* vesicles and filaments (B).
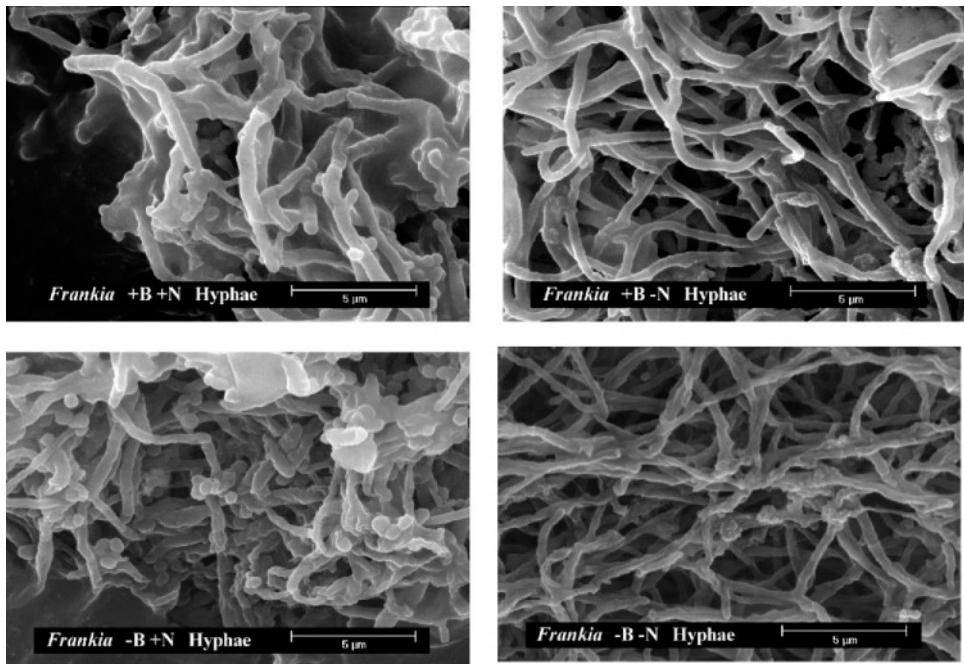
Figure 5. Scasnning electron microscopy study of filaments of *Frankia* BCU110501 grown in media containing combined nitrogen (+N) or under nitrogen fixing conditions (–N), and in the presence (+B) or in the absence (–B) of boron. Boron deficiency leads to thinner twisted filaments.

3. ROLE OF BORON IN NITROGEN FIXING SYMBIOSES

With the exception of the symbiosis established by cyanobacteria, endophytic N_2-fixing symbioses involve the development of a new plant organ, generally in the root, the nodule. This provides an ecological niche for the endophytic bacteria. Most important root nodule symbioses are established by eubacteria of the family *Rhizobiaceae* with leguminous plants or by actinomycetes of the genus *Frankia* with certain non-leguminous shrubs and trees termed actinorhizal. Both rhizobial and acti-norhizal symbioses are induced by an exchange of signals and the development of both types of root nodules implicates bacteria-plant interactions that lead to sym-biotic-specific differentiations of the partners (Pawlowski and Bisseling, 1996). New synthesis and deposition of wall and membrane material have to be carried on to build a nodule; and several bacteria and plant macromolecules decorated with cis-diol rich glycosil-moieties are implicated in plant-bacterial cell surface interactions. Therefore, B is a clue element in the establishment and maintenance of these sym-bioses.

3.1. Legume-rhizobia symbiosis

The requirement of B for symbiotic N_2 fixation in legumes was suggested by Brenchley and Thornton (1925). They reported low number and non-functional

nodules in B deficient *Vicia faba*. Since B is apparently not essential for *Rhizobium* growth, these authors attributed the alterations to an effect of B deficiency on the vascular tissue, which would not allow a normal transport of nutrients from root to nodule. These results were corroborated 7 decades later in nodulated *Pisum sativum* (Bolaños et al., 1994) and *Phaseolus vulgaris* (Bonilla et al., 1997a) plants. In both cases, B starvation resulted in a reduction of nitrogenase activity of about a 50% after 2 weeks of treatment and about a 70% after 3–4 weeks post-inoculation with *Rhizobium*.

Measurements of tissue distribution of B in legume plants show that the micronutrient accumulates in nodules more than in other plant organs. Such a high B requirement gives rise to the idea that B is involved not only in vascular tissue maintenance, but also in the establishment of functional rhizobial symbiosis.

During nodule development, an extensive synthesis of membrane of about 30–50 fold that given in other tissues occurs in infected cells, to build the peribacteroid membrane of each symbiosome (Robertson et al., 1984; Bradley et al., 1986) (see below). Since most of B in plants is bound in cell walls (Thellier et al., 1979) and membranes (Torchia and Hirsch, 1982; Parr and Loughman, 1983), it is logical to find high levels of B in nodules. Furthermore, plant-derived glycoconjugates or the glyco-components from the cell surface of *Rhizobium* play an essential role in the correct establishment of the symbiosis between legumes and rhizobia (see Kannenberg and Brewin, 1994 and references therein). Most of these molecules contain cis-diol groups able to interact with borate anions. Therefore, not only the stabilisation of nodule cell wall and membrane structure but also the maintenance of a correct bacteria-plant language can be expected to be roles of B in legume-rhizobia N_2-fixing symbiosis.

Most important studies on the incidence of B in the different stages of the legume-rhizobia symbiosis and nodule development are reviewed below.

Table 3. Effects of boron nutrition on nitrogenase (acetylene reduction) activity expresed as nmol C_2H_4 plant^{-1} h^{-1} of *Pisum sativum* inoculated and *Phaseolus vulgaris* plants inoculated with *Rhizobium*.

	+B (9.3 µM B)	−B (no added B)
Pisum sativum	166 ± 33	43 ± 11
Phaseolus vulgaris	646 ± 72	190 ± 37

Table 4. Boron content (µg g^{-1} dry weight) in different plant organs of nodulated *Pisum sativum* grown in the presence or in the absence of B.

	+B (9.3 µM B)	−B (no added B)
Shoot	33.12 ± 5.90	15.62 ± 4.32
Root	25.35 ± 3.61	8.38 ± 2.49
Nodule	43.53 ± 3,45	3.87 ± 0.23

3.1.1. *Nodule structure and function*

Nodules developed in the absence of B are smaller in size and in weight than nodules with B. Most of nodules from low B plants appear pale in contrast with the bigger pink normal nodules as reflect of the absence of the oxygen carrier leghemoglobin in –B nodules (Figure 6). This indicates that these nodules are not functional.

Typical symptoms of B-deficiency appear in the structure of those nodules developed without B. Most of the cells appeared enlarged and irregularly shaped. There is no evident differentiation between nodular tissues (infected zone and inner and outer cortex). Cell walls present some regions ticker than normal and others thinner or even without wall deposition in B-deficient nodules. In addition, cell wall and membrane breakage also takes place in B-deficient nodules.

Studies at a molecular level indicate that several components of the cell walls of B-deficient nodules are abnormally assembled, leading to aberrant walls. Bean nodules devoid of B have walls without covalently bound hydroxyproline-/proline-rich glycoproteins (Bonilla et al., 1997a), which are developmentally regulated during nodule growth (Cassab, 1986). Particularly, a protein similar to the product of the early nodule specific protein (nodulin) (ENOD2) gene is absent in the cell walls of the nodule parenchyma in bean plants. These nodulin could belong to the extensin
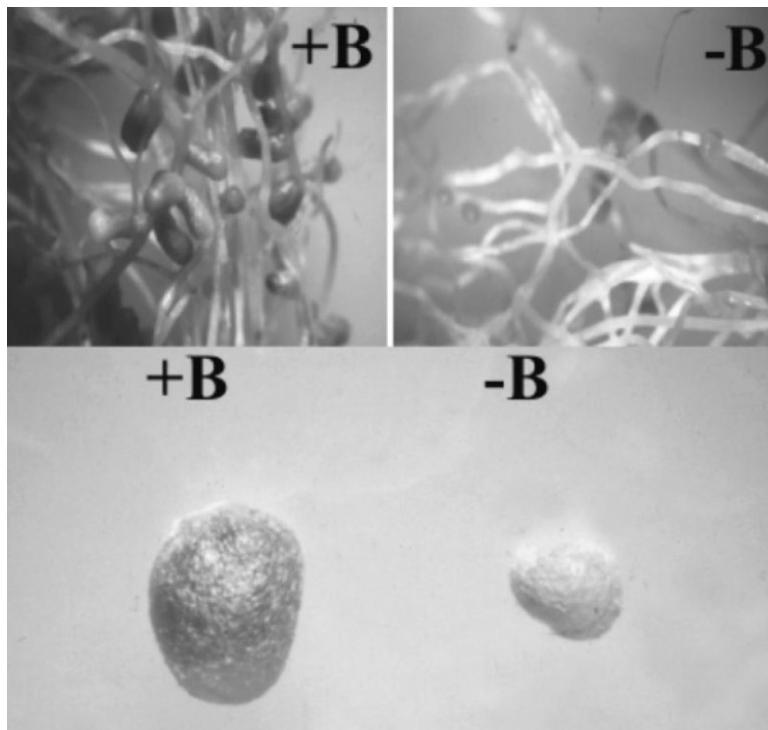

Figure 6. Effects of boron deficiency on root and nodule development in *Pisum sativum* 3 weeks post-inoculation with *Rhizobium leguminosarum*. In bottom side, there is a higher magnification of a +B and a –B nodule that illustrate differences of development due to boron.

family of the cell wall (Kieliszewski and Lamport, 1994). Nevertheless, northern analysis shows that ENOD2 mRNA is still present in B-deficient bean nodules, indicating that the expression of the ENOD2 gene is not affected by the lack of B, but the assembly of the protein into the cell wall is.

Besides wall proteins, changes in the contents of the cell wall pectin polygalacturonan either as O-methyl esterified or unesterified molecule have also been found (Bonilla et al., 1997b).

3.1.2. *Plant-bacteria signalling and preinfection events*

The N_2-fixing legume root nodule is the result of genetically determined interactions between rhizobia and the host plant (Stougaard, 2000). The exchange of diffusible signal molecules between both partners results in the activation of rhizobial *nod* (nodulation) genes in response to flavonoids in root exudates (Spaink, 2000). The products of *nod* gene activity are the Nod (nodulation) factors, lipochitin-oligosaccharides that induce root hair deformation, cortical cell division (Dénarié and Cullimore, 1993) and preinfection structures in curled root hairs (van Brussel et al., 1992; van Spronsen et al., 2001) in the appropriate host legume.

Nodulation is reduced more than a 50% in the absence of B, because the micronutrient is implicated in the signalling process (Redondo-Nieto et al., 2001). Root exudates from plants grown without B stimulated *nod* gene expression at a level very low compared with exudates derived from root plants grown with B. The curling and deformation of emerging and growing root hairs in response to Nod factors secreted by *Rhizobium* is therefore altered in B-deficient pea plants, which showed a very low root hair deformation rate compared with control plants 3 days after inoculation.

These effects might be reflect of the phenolics and hence flavonoids metabolism. Boron nutrition has an effect on the activity of key enzymes in the metabolism of phenolics (Fawzia et al., 1994; Ruiz et al., 1998), and changes in flavonoids implicated in defence against insects have been reported in B-deficient plants (Rajaratman and Hock, 1975). Similarly, B deficiency can also modify the presence or release of flavonoid compounds that induce the expression of nodulation genes. In response, the secretion of Nod factors by the host *Rhizobium* is reduced.

Besides diffusible signals, a second type of preinfection interaction involving the attachment of rhizobia to roots is needed to initiate nodule formation on pea (Kannenberg and Brewin, 1994). The study of root colonization by rhizobial cells indicated that B deficiency in pea plants also diminishes the physical interaction between the host roots and *Rhizobium* (Redondo-Nieto et al., 2001). The role of B in the maintenance of plant cell wall structure is very well established (Blevins and Lukaszewski, 1998; O'Neill et al., 2001) and therefore, changes in the structure of the B-deficient cell surface can be responsible for the low capacity of adsorption of bacteria.

The inhibition of both signalling and root colonisation processes by B deficiency justifies the reduction of the amount of nodules developed in B-deficient legumes.

3.1.3. *Infection threads development and cell invasion*

In roots of legumes as *Pisum, Medicago, Trifolium*, or *Vicia*, the cell division is induced by Nod factors in the inner layers of the root cortex. Meanwhile, rhizobia make contact with the plant cell surface and invade the plant through a transcellular tunnel (the infection thread) sheathed with cell wall material (Rae et al., 1991). A direct interaction between the plant and the bacteria cell surfaces seems to play a part in the formation of infection threads. Within the threads, rhizobia are embedded in intercellular plant derived matrix material, including a plant matrix glycoprotein (MGP), recently identified as a new extensin-like glycoprotein (Rathbun et al., 2002), that is secreted by plant cells into the lumen of the infection thread as an early response to rhizobial infection (VandenBosch et al., 1989; Rae et al., 1992). Rhizobia invade and spread from cell to cell by growth and ramification of infection threads followed by bacterial release from an unwalled infection droplet that extrudes from the thread into the host cytoplasm (Brewin, 1991). This endocytosis process seems to require an infection droplet membrane-rhizobia cell surface interaction, possibly mediated by glycolipids and/or glycoproteins of the plant membrane and the lipopolysaccharide component of the bacterial outer membrane (Bradley et al., 1986). At the same time, other cells are also stimulated to divide forming a persistent apical meristem generating a cylindrical indeterminate nodule.

This sequence of cell division and cell invasion varies in other legumes as *Phaseolus, Glycine* or *Lotus*. The cortical cell division starts in the outer cortex near the infected root hair. These cells are invaded through infection threads before they become meristematic (Rolfe and Gresshoff, 1988). Rhizobia can then spread by division of those infected meristematic cells. At the same time, a new centre of cell division originates in the inner cortex and forms an envelope that differentiates into nodule cortex and the vascular bundles (Taté et al., 1994). This gives rise to a spherical determinate nodule in which meristematic activity is transient.

Infection threads in B-deficient legumes are extremely enlarged and aborted prematurely (Bolaños et al., 1996), even in the root hair previous to reach the cortical cell (Redondo-Nieto et al., 2001). Furthermore, both indeterminate (pea) and determinate (bean) nodules appear almost uninvaded when they are induced in the absence of B (Bolaños et al., 1994; Bonilla et al., 1997a). This might be due to a role of B as modulator of the interactions between the plant derived infection thread matrix glycoprotein (MGP) and the bacteria cell surface. In the absence of B, the MGP can attach to the cell surface of rhizobia. Therefore, the bacterium can be trapped and unable to interact with the plant cell membrane and hence elicitation of the endocytosis process is inhibited as illustrate the model of Figure 7B. The presence of B (but not Ca, pH changes, salt or high ionic strength) specifically inhibits the *in vitro* bacteria-MGP attachment and promotes the rhizobial interaction with the plant membrane (Figure 7A) (Bolaños et al., 1996). As a result of this effect of B deficiency, the infection threads development arrests at an early stage prior endocytosis (Redondo-Nieto et al., 2001), leading to poorly invaded nodules similar to those shown in Figure 8.
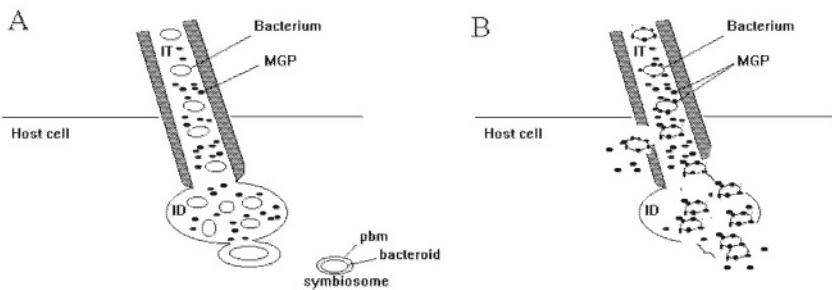
Figure 7. Model for the effect of B on cell invasion by *Rhizobium* through infection threads (IT) and droplets (ID). In the presence of B (A), binding of the infection thread matrix glycoprotein (MGP) to the cell surface of the bacterium is prevented; once in the infection droplet, the interaction between *Rhizobium* and the plant cell membrane promotes endocytosis. In the absence of B (B), MGP binds to the bacterial cell surface and prevents the subsequent plant membrane-*Rhizobium* interaction and endocytosis; invasion comes through breaks of cell wall and membrane degenerated by B deficiency.
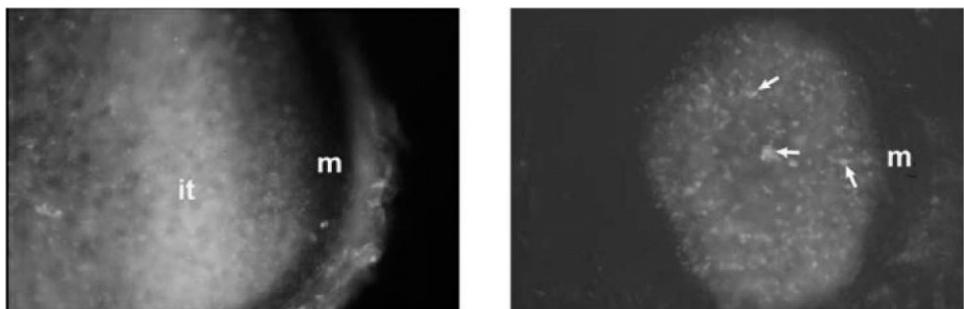
Figure 8. *Pisum sativum* root nodules induced by a *Rhizobium* strain that constitutively expresses green fluorescent protein (GFP). Fluorescence of nodules developed in the presence of boron (+B) reveals a central infected tissue (it) with cells full of bacteria. In B-deficient nodules (–B) green fluorescence appear only in enlarged infection threads (arrows) and cells appear empty of bacteria. (m) nodule meristem.

3.1.4. *Symbiosome development*

Endophytic rhizobia are engulfed by plasma membrane and come to occupy an organelle-like compartment, termed the symbiosome. Intracytoplasmic bacteria, termed bacteroids, proliferate and eventually develop the capacity for fix nitrogen (Brewin, 1991). Bacteroids are enclosed by a plant-derived peribacteroid membrane (PBM) that harbours a differentiated form of plasma membrane glycocalyx composed of a mixture of glycoproteins and glycolipids (Perotto et al., 1991). Between the PBM and the bacteroid there is a peribacteroid fluid (PBF), which is lysosomal in character (Mellor, 1989) and contains glycoproteins, including specific nodule lectin-like glycoproteins (Kardailsky et al., 1996). During the symbiotic interaction the structure of the symbiosome components differentiate closely synchronized (Verma, 1992). Maturation implies gradual differentiation of the PBM at structural (Miao et al., 1992; Perotto et al., 1995) and functional (Day and Udvardi, 1993) levels from

those of the plasma membrane, targeting of proteins to the PBF (Mellor, 1989), and bacteroid development to a N_2-fixing form (de Maagd et al., 1994; Kannenberg et al., 1994).

Symbiosomes appear with a degenerated peribacteroid membrane (PBM) and a complete alteration of bacteroid structure in B-deficient nodules (Bolaños et al., 1994) (Figure 9). Several plant and bacterial glycoconjugates able to interact with B are implicated in this phenomenon (Kannenberg and Brewin, 1994). The study of PBM-glycoproteins and -glycolipids revealed that most of components from the PBM disappeared in mature B-deficient nodules, due to membrane degradation (Bolaños et al., 2001). Besides membrane degradation, most important differences during nodule development in the absence of B are found in the PBF glycoproteins in pea (Bolaños et al., 2001). During symbiosome maturation new proteins are targeted to the PBF. The only components identified at the moment are two isoforms of a nodule specific lectin-like glycoproteins (*Pisum sativum* nodule lectin, PsNLEC-1) These components seem to be implicated in bacteroid maturation since pea mutant that not express the two symbiosomal isoforms of Ps-NLEC-1 harbours contain bacteroids that arrest at an early stage of differentiation (Brewin et al., 1995; Dahiya et al., 1998). Most glycoproteins disappeared and PsNLEC-1 glycoproteins are never detected in B-deficient symbiosomes. The detection by specific antibodies of sugar groups of PsNLEC-1 demonstrated that the carbohydrate-moiety of this protein was modified in the absence of B. Localization in ultra-thin pea nodule sections of PsNLEC-1 glycoproteins, which appeared localized in the PBF compartment of infected cells showed that they were accumulated in Golgi-derived or cytoplasmic vesicles in B-deficient nodules. This indicates a failure of the targeting of Ps-NLEC glycoproteins to the PBF of symbiosomes in B-deficient nodules.

A role for B in the targeting of vesicles containing glycoproteins has already been proposed in other plant tissues (Goldbach, 1997). Boron can mediate bridging between hydroxyl groups (mainly mannose moieties of glycoproteins) of ligands in vesicles and membrane promoting membrane fusion and the subsequent release of the vesicle content. The targeting of vesicles to the symbiosome compartment can
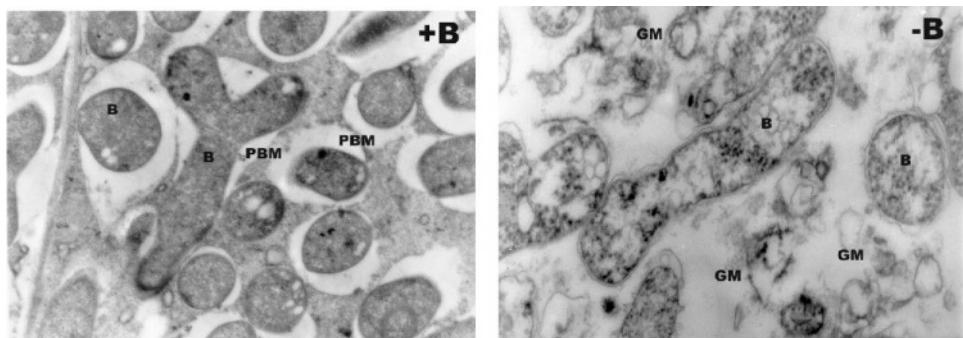

Figure 9. Effects of boron deficiency on symbiosome development. In the presence of boron (+B), bacteroids (B) appear surrounded by a peribacteroid membrane (PBM). In B-deficient nodules (–B), the PBM is degraded and only ghosts of membrane (GM) are visible.

be arrested due to the lack of B bridges and/or the absence of the proper hydroxylic ligand, which may be lost during the abnormal glycosilation in B deficiency.

3.2. Actinorhizal symbiosis

As above described, B is essential for growth and nitrogen fixation in free-living *Frankia*. The absence of the micronutrient during the establishment of the symbiotic relationship between the actinomycete and its host actinorhizal plant affects negatively the symbiosis (Bolaños et al., 2002a, Bonilla et al., 2002). *Discaria trinervis* plants inoculated with *Frankia* BCU110501 grown in N-free media and in the absence of B present a diminished nodulation rate. The number of developed nodules per plant was halved in B-deficient plants and these nodules were non-functional. This negative effect is still amplified when the inoculum comes from a B-deficient culture of *Frankia*, due to the essentiality of B for the micronutrient, being nodulation rate reduced to a 10%.

Although little is known about *Frankia*-actinorhiza molecular signalling and cell surface interactions, these observations reflect that B could modulate them, as it does during the *Rhizobium*-legume relationship.

Growth of plants is obviously related with the behaviour of nodulation of each treatment. The size, root weight, and shoot weight showed a significant reduction in poorly nodulated *Discaria*, including B-deficient plants or plants inoculated with B-deficient bacteria. Therefore, both the plant and *Frankia* require B for a correct establishment of a symbiotic relationship.

4. CALCIUM AND B-Ca RELATIONSHIP IN NITROGEN FIXING SYMBIOSIS

The study of the interaction between both B and Ca is an important topic in plant mineral nutrition. There are several common features of both nutrients (i.e. low mobility, higher extracytoplasmic concentration, growth alterations during deficiency . . .). The amount and availability of one of these nutrients influences the distribution (Ramón et al., 1990) and the requirements of the other for optimal plant growth (Teasdale and Richards, 1990). Actually, the Ca/B ratio was very soon proposed as an indicator of plant nutritional status (Brennan and Shive, 1948).

Evidence of a B-Ca interaction for cell membrane function has been reported (Tang and De la Fuente, 1986), although most investigations on the topic dealt with the structure and function of the cell wall. In a chemical study, Van Duin et al. (1987) described that Ca^{2+} is able to form complexes with borate-polyhydroxycarboxylates through direct interaction with the borate anion. O'Neill et al. (1996, 2001) reported that the rhamnogalacturan II region is stabilized by B through borate-diester bonds with apiosyl residues. Furthermore, Kobayashi et al. (1999) demonstrated that Ca^{2+} promoted *in vitro* formation of dimmers of borate-rhamnogalacturan II and proposed that Ca^{2+} stabilizes pectin polysaccharides of the cell wall through ionic and coordinate bonding in the polygalacturonic acid region.

Regarding rhizobia-legume symbiosis, Carpena et al. (2000) reported that addition

Table 5. Boron content (μg g^{-1} dry weight) in nodules of *Pisum sativum* grown with different concentrations of B and Ca^{2+}.

+B (9.3 μM) +Ca^{2+} (0.68 mM)	−B +Ca^{2+} (0.68 mM)	−B +2Ca^{2+} (1.36 mM)
43.53 ± 3.45	3.87 ± 0.23	18.21 ± 2.36

of high Ca during pea growth under B deficiency could mediate mobilisation of B from old to young tissues in nodulated pea plants. In agreement with this report, measurements of the concentration of B in nodules developed in B-deficient plants indicate that it is higher when plants grow with a supplement of extra Ca. This is a sign of a B-Ca interaction also in the establishment and maintenance of the symbiosis.

Previous to summarised what is known about this subject, it is interesting to point out the importance of Ca in plants and for nitrogen fixation either in free-living cyanobacteria or in the legume-rhizobia relationship.

4.1. Calcium in plants

The traditional functions of calcium in plants revolved around cell wall structure, and membrane structure and function (Leonard and Hepler, 1990). However, most recent reviews are focused on calcium ions as one of the most important messengers involved in signal-response coupling (Rudd and Franklin-Tong, 2001; Sanders et al., 2002). Several physiological processes are accompanied with changes in cytoplasmic calcium concentration (Trewavas and Malhó, 1998). Moreover, a number of external stimuli led to changes in cytosolic Ca^{2+} (Bush, 1995). In prokaryotic cells, an equivalent important role for Ca^{2+} has been hard to demonstrate, but it is now becoming clear (Smith, 1995; Norris et al., 1996).

However, the role(s) of calcium is (are) still not well defined. To demonstrate a regulatory role of Ca^{2+} in any cell systems, it is essential to measure resting intracellular free Ca^{2+} levels, as well as those arising in response to stimuli or environmental signal; nevertheless, accurate quantification during cellular signalling events has proven very difficult, because cytosolic Ca^{2+} is at micromolar concentrations, and spikes in response to external changes sometimes occur very quickly. Fortunately, the possibility of transforming plant, animal, and bacterial cells with the Ca^{2+}-binding-sensitive luminescent protein apoaequorin has allowed the quantification of intracellular Ca^{2+} fluxes accompanying diverse stimuli (Knight et al., 1991a, b; Takahashi et al., 1997; Gong et al., 1998).

4.2. Role of calcium in nitrogen fixing cyanobacteria

The possible regulatory role of calcium in cyanobacteria by measuring intracellular free Ca^{2+} levels has been investigated in a recombinant strain of the nitrogen fixing cyanobacterium *Anabaena* PCC7120, which constitutively expresses the Ca^{2+}-binding-protein apoaequorin. This system allows the study of the homeostasis of intracellular Ca^{2+} levels in this cyanobacterium and to monitor Ca^{2+} transients in

response to environmental stresses such as heat and cold shock (Torrecilla et al., 2000), salinity and osmotic stress (Torrecilla et al., 2001).

Regarding nitrogen fixation, a high Ca^{2+} requirement for cyanobacteria related to the resistance to oxygen of heterocysts has been reported (Rodriguez et al., 1990; Gallon, 1992). Moreover, calcium has been implicated in the stability of heterocysts envelope and consequently in the protection of nitrogenase activity under stress conditions (Fernández-Piñas et al., 1995).

4.3. Role of calcium in legume symbiosis

Impaired N_2-fixation in legumes due to Ca deficiency was reported by Greenwood and Hallsworth (1960). Later, Lowter and Loneragan (1968) described that high Ca supply was required to induce a high number of nodules in the plants, and Munns (1970) described a higher Ca requirement for early infection events. These studies indicate a role of Ca for plant-bacteria signalling and recognition. Moreover, the importance of Ca^{2+} in signal transduction during nodule organogenesis is also an important research topic.

4.3.1. *Early interactions*

The activity of *nod* genes is higher when the amount of Ca for plant growth increases. Richardson et al. (1988) demonstrated that high Ca increased the amount of *nod*-gene inducing compounds in root exudates. This effect can be due to the role of Ca on the synthesis of flavonoids. Application of external Ca to plants increases the PAL (phenylalanine ammonia-lyase) activity (Castañeda and Pérez, 1996), the key enzyme in the flavonoid synthesis pathway.

Calcium is also required for bacterial attachment to the root hair (Lodeiro et al., 1995). Among others, this interaction is mediated by plant and bacterial components able to use Ca^{2+} as a ligand to reinforce the attachment. Calcium ions can therefore strengthen the activity of plant lectins or rhizobial Ca^{2+}-dependent ricadhesines (Smit et al., 1989). Moreover, bacterial exopolysaccharide (EPS) can form a gel in the presence of cations as Ca^{2+}, being a non-specific mechanism for rhizobial attachment (Morris et al., 1989).

4.3.2. *Signal transduction*

Calcium has also been demonstrated to act as a second messenger in the Nod-factor signal transduction (see Cárdenas et al., 2000; Lhuissier et al., 2001; and refs. threrein). The first detectable event after Nod factor application is an influx of Ca^{2+} at the root hair tip (Felle et al., 1998). This could lead to an efflux of Cl^- and membrane depolarisation (Downie and Walker, 1999), causing an increase of cytosolic Ca^{2+} within a few minutes at the root hair tip (Cárdenas, et al., 1999; Felle et al., 1999). The actin cytoskeleton reacts to Nod factors within 3 min of their application (Cárdenas et al., 1998). The cascade involved in the transduction of Nod factor signalling is mediated by a G-protein and phospholipases C (PLCs) (Pingret et al., 1998) that are fully activited by Ca^{2+}. The organization and function

of actin filaments is highly determined by regulatory actin binding proteins (ABP) that have sites for phospho-inositide (IP) binding. Hydrolysis of phosphatidylinositol biphosphate (PIP_2) by PLCs produces water soluble IP_3 that can therefore regulate ABP-mediated rearrangements of actin filaments and bundles. This is consistent with the idea that elevated cytosolic Ca^{2+} causes re-organization of the cytoskeleton at the root hair. The other changes induced by Nod factors that depend upon changes in actin filaments, including root hair tip swelling, vacuolation, endoplasmic reticulum alignment with the plasma membrane, nuclear movement to the swelling, and inward growth of cell wall can be related to these Ca^{2+} dynamics.

Furthermore, there are other later Ca^{2+} spikes originating from the perinuclear region of root tip approximately 9 min after Nod factor application and that extend for at least 60 min to 3 h (Ehrhardt et al., 1996). The initiation of cytosolic Ca^{2+} elevation implicates mobilization of internal Ca^{2+} stores, possibly from endoplasmic reticulum, mediated by PLC-produced IP3 (Muir and Sanders, 1997). Although the role of these spikes is still unclear, there is some information concerning gene expression (Schultze and Kondorosi, 1998; Felle et al., 1999) that is important for cell cycle regulation during nodule organogenesis.

4.4. B-Ca interaction in nitrogen fixation

Similarly to the investigation of the roles of boron on nitrogen fixation, there are several studies that demonstrated a relationship between the micronutrient and calcium in free-living N_2-fixing heterocystous cyanobacteria, but also in non-fixing strains, which do not require B for growth under normal conditions. Besides, nodulation and the different steps of nodule development and organogenesis in the legume-rhizobial symbiosis are highly influenced by both nutrients.

4.4.1. *B-Ca relationship in cyanobacteria*

It has been reported that calcium prevents the damage of structures in boron deficient heterocysts, and hence restores their functionality (Bolaños et al., 1993). Since a reciprocal interaction between B and Ca^{2+} is possible, boron could recover Ca^{2+} deficiency in a manner similar to calcium under B-deficient conditions, indicating a co-operative role of B and Ca^{2+} in cyanobacteria (Bonilla et al., 1995). The response to boron supplementation of Ca^{2+}-deficient *Synechococcus* cultures suggests that, although under normal conditions B is not essential for non N_2-fixing cyanobacteria, this micronutrient could play a role when these microorganism are grown under calcium limiting conditions. Consequently, *Anabaena* and *Synechococcus* growth in media without added calcium could partially recover after addition of B. As in *Anabaena*, boron supplementation restored photosynthesis and chlorophyll content. Uptake of nitrate also showed a B-mediated recovery. These results support the hypothesis that boron might facilitate the uptake of calcium. Therefore, boron is required for growth under certain conditions by groups of organisms that have been reported to not require the micronutrient.

4.4.2. *B-Ca relationship in nitrogen fixing symbiosis*

Not only in tissue distribution, but also in nodulation and in nitrogenase activity of legume nodules the relationship between B and Ca can be clearly stated (Bolaños et al., 2002b).

Pea, bean, and alfalfa plants grown in media containing different concentrations of B and Ca, and inoculated with their host rhizobia develop different amount of nodules and nitrogen-fixing activity depending of the level of both B and Ca in the growth media.

Deficiency of the micronutrient resulted in a high inhibition of nitrogenase activity, and significant toxic effects on nitrogen fixation appear at concentrations of B as low as 0.5 ppm. The addition of extra Ca to B-deficient legumes recovers nodule function, but only partially (Figure 10, nitrogenase activity). Alternatively, the decrease of Ca also mitigates the effects of B toxicity. In treatments where low Ca was added to the nutrient solutions, the nitrogenase activity is highly inhibited, but the increase of B resulted in a small recovery.

Moreover, nodulation always increases in plants treated with a high level of Ca, even in B-deficient plants (Figure 10, nodule number). As stated above, B is implicated in almost any event of the legume-rhizobia symbiosis, while Ca is especially required for early preinfection events. This explain that nodulation seems to depend of Ca more than of B, since Ca supplemented plants develops a higher number of nodules at any tested concentration of B.
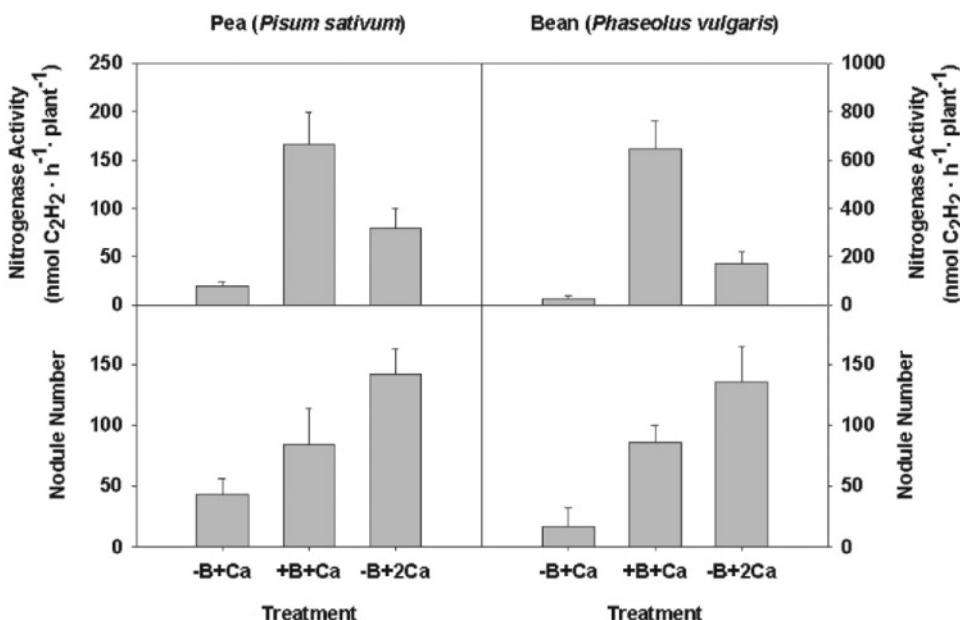

Figure 10. Effects of B (0 to 9.3 μM) and Ca^{2+} (0.68 to 1.36 mM) concentrations on nitrogenase (ARA) activity and nodulation (nodules per plant) of *Pisum sativum* (pea) or *Phaseolus vulgaris* (bean) plants.

4.4.2.1. *Nodulation and nodule development*

Determination of *nod* gene activity, which is very low after exposure of *Rhizobium* to root exudates derived from B-deficient plants, demonstrates a higher induction capacity in plants treated with high concentrations of Ca. Low B leads to an increase of PAL activity (Ruiz et al., 1998), but to a reduction of flavonoids, because B deficiency also increases the activity of peroxidase and polyphenoloxidase from the oxidative pathway of phenolics to quinones (Marschner, 1995). However, Ca increases PAL activity and diminishes peroxidase and polyphenoloxidase activities (Kawai et al., 1995; Tomasbarberan et al., 1997), leading to a higher production of flavonoids. Therefore, there might be an increase of *nod* gene-inducing flavonoids in high Ca treated roots even under B deficiency.

Besides the exchange of diffusible signals, Ca can also recover the adsorption of rhizobia to roots that is needed to initiate the formation of a nodule.

Both phenomena may explain why Ca is more important and can replace B at early preinfection stages of nodule development.

Following nodule development, rhizobia must induce infection thread development and endocytosis (Brewin, 1991). The presence of high Ca during plant growth recovered the phenomenon of cell invasion, which is aborted in B-deficient nodules (Bolaños et al., 2002b). This effect could be due to the inhibition of the attachment of the MGP the cell surface of rhizobia by Ca, similarly to occurring with B. However, borate ions, but never Ca, prevent specifically this interaction. This suggests that Ca cannot directly recover the progression of bacteria through infection threads and the process of invasion. Carpena et al. (2000) reported that addition of high Ca during pea growth under B deficiency could mediate mobilisation of B from old to young tissues. The concentration of B in B deficient nodules is actually higher when plants growth with a supplement of Ca, as previously shown in Table 5. Therefore, B redistributed by high Ca could be the responsible of the recovery of nodule invasion in –B nodules treated with extra Ca.

4.4.2.2. *Nodule cell wall structure*

Addition of calcium can therefore restore most effects of B deficiency during the development of the root nodule. However, nitrogenase is only partially restored. This indicates that B is absolutely essential for the proper functioning of nodules and that Ca cannot replace the micronutrient in this function.

The explanation can be found in the structure of nodule cell walls. Boron is absolutely required for the proper organisation of the cell walls, and extra Ca is not able to fully recover the structure of B-deficient walls (Bolaños et al., 2002b). The *in vitro* studies by Kobayashi et al. (1999) demonstrated a B-Ca interaction in the stabilisation of pectin polysaccharides. In agreement with these studies, the unorganised pectin fraction of B-deficient nodule cell wall is not stabilised by Ca, which indicates that B and Ca play a complementary role and that both are essential for proper nodule structure and function.

5. B-Ca RELATIONSHIP IN THE ADAPTATION OF
LEGUME SYMBIOSIS TO SALINITY

As cited above, the Ca/B ratio can be an indicator of plant nutritional status. The relationship is important for legume symbiosis and nitrogen fixation not only under physiological conditions, but also under stress. Our group has recently initiated a new research line that studies the role of the B/Ca ratio in stress tolerance. This can be a very easy and cheap tool to increase crop production in adverse soils.

5.1. Nitrogen fixation under salt stress

Among the adverse soil conditions for agricultural systems, salinity has been a factor that has influenced even the establishment of human populations. Nearly 50% of world's irrigated land is categorised as having potential salinity problems (Rhoades and Loveday, 1990).

There are two main negative effects of high salt concentrations that influence plant growth and development: water deficit (Munns and Termaat, 1986) and ion toxicity associated with excessive Cl^- and Na^+ (Niu et al., 1995). This results in nutrient imbalance that leads to Ca^{2+} and K^+ deficiency (Cramer et al., 1987) and to other nutrients imbalance (Marschner, 1995 and refs. therein). Plants differ greatly in their response to salinity (Hasegawa et al., 2000), and most legumes are classified as salt sensitive crop species (Greenway and Munns, 1980; Lauchli, 1984).

Biological nitrogen fixation offers a great agronomic interest. It is estimated that rhizobial symbiosis with over one hundred of agriculturally important legumes account, at least for half of the annual amount of nitrogen fixation in soil ecosystems (Peoples and Craswell, 1992). Moreover, the use of N as fertiliser has degraded huge land extensions around the world and biological nitrogen fixation is required to replace tonnes of fertilisers (Burris, 1994). Therefore, symbiotic nitrogen fixation in legumes is particularly important both agriculturally and ecologically, and studies to guarantee the success of the *Rhizobium*-legume symbiosis even under severe environmental conditions are crucial for its application in arid zones.

Nodulated leguminous plants are singular because they have the specialised root nodule where the N_2-fixing process takes place, which is the product of molecular interactions between the host plant and the rhizobia. Therefore, salt stress affects the macro-, the microsymbiont and nodule development, structure and function. Studies of tolerance to salt indicate that the plant is usually less tolerant to the stress than the microsymbiont (El-Shinnawi et al., 1989; Zahran, 1991), and sometimes legumes are more sensitive growing in symbiosis than with N fertiliser (Lauchli, 1984). The responses of rhizobial strains to high salt include accumulation of ions as K^+ and low-molecular weight compounds called osmolites (Bostford and Lewis, 1990), and changes in the surface polysaccharides (Lloret et al., 1995, 1998; Zahran et al., 1994).

The effects of salt stress on nitrogen fixation in legumes have been widely reported (for a review: Zahran, 1999). High salt can directly impair the interactions between *Rhizobium* and the host plant inhibiting nodule formation (Singleton

and Bohlool, 1984). Salinity can also indirectly affect the symbiosis by reducing the growth of the host plant.

5.2. B-Ca interaction in legume-rhizobia symbiosis under salt stress

Osmotic stress or ionic imbalance may cause disorders in almost any physiological process, and mechanisms of salt-tolerance in plants are genetically determined (Hasegawa et al., 2000; Zhu, 2001). Therefore, the selection of host legume genotypes that are tolerant to high salt conditions is important to determine the success of the rhizobial symbiosis (Cordovilla et al., 1995; Velagaleti and Marsh, 1989).

Complementarily, the study of the interaction among nutrients that are especially required for nodulation, such as boron and calcium, with salt is important to optimise the conditions for salt tolerance of inoculated legumes. As cited above, Ca^{2+} deficiency is a typical feature of salt stress, and a reduction of B concentration due to salinity has also been reported (El-Motaium et al., 1994). Therefore, the legume nodule might be especially affected by this nutrient imbalance. The addition of Ca to legumes grown under high NaCl concentrations had positive effects on nitrogen fixation (Akhavan-Kharazian et al., 1991), however, a wide study of the interaction B-Ca related to salt tolerance is still to be developed, in spite the importance of the B/Ca relationship.

5.2.1. *Effects of salt stress on growth, nodulation and nitrogen fixation of symbiotic pea plants*

The limiting salt level for pea (*Pisum sativum* cv. Argona) and its host bacteria *Rhizobium leguminosarum* bv. *viciae* strain 3841 was 75 mM NaCl (El-Hamdaoui, 2002). At that salt concentration, the development of plants was severely diminished. Nodulation, measured as number of nodules, and nitrogen fixation, measured as acetylene reduction activity (ARA), were almost completely inhibited by 75 m*M* NaCl. As a result of low nitrogen fixation, the N-content of salt treated plants was also lower than in control (without salt) plants. These results indicate that nitrogen-fixing pea plants are very salt-sensitive.

Nodules from pea roots developed in the absence of salt presented the typical structure of indeterminate nodules. This included a meristematic tip region with small cells and large regularly shaped cells from the central to the root zone of the nodule. By contrast, nodules developed under salt stress appeared with a very altered structure, without tissue differentiation, and cells were very irregularly shaped.

5.2.2. *Effects of B and Ca nutrition on the development and nitrogen fixation of salt treated symbiotic pea plants*

Growth of inoculated pea plants growing under salt stress can be enhanced by an adequate nutrition of B and Ca^{2+}. Increases of Ca concentrations recovered partially plant development, as typically reported (LaHaye and Epstein, 1971). However,
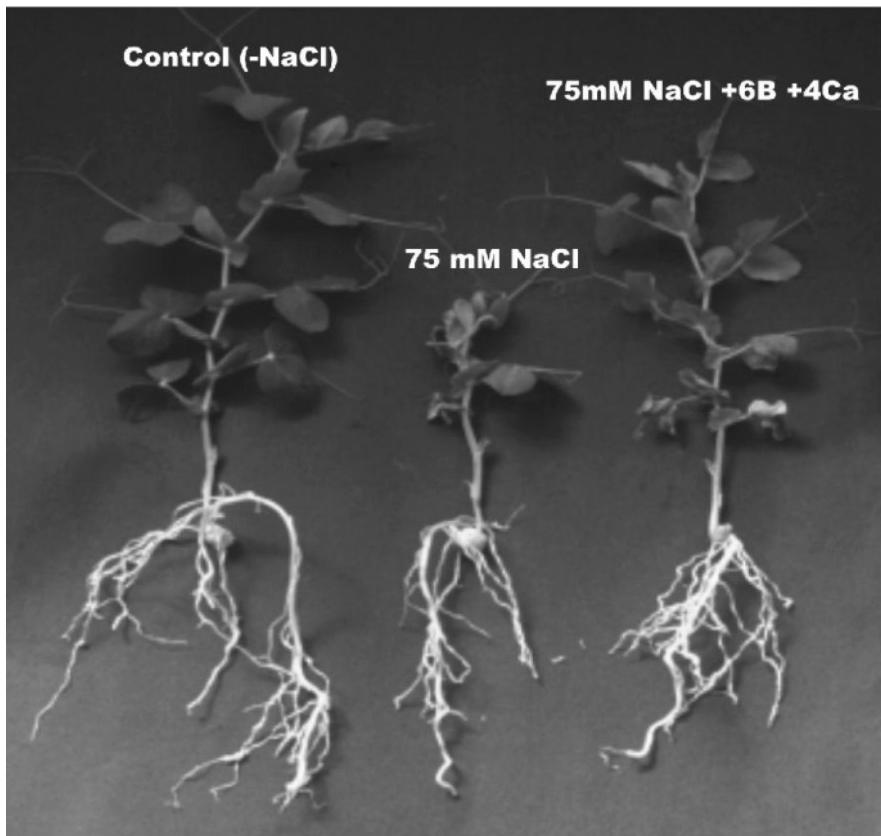
Figure 11. Effects of salt stress and increased levels of B and Ca on the development of *Pisum sativum* nodulated plants.

combination of increased B and high Ca concentrations produced the best recovery effects on shoot and root development in plants grown in saline media.

Nodulation and N_2 fixation of plants grown under salinity could also be recovered by modifications of B and Ca. The increase of Ca increased the amount of nodules per plant, and that was an effect independent of the concentration of B. However, Ca was not enough to restore nitrogen fixation, and the addition of extra B was essential to partially recover nodule function.

The study of the structure of nodules developed under high salt and with different B and Ca treatments also shows a beneficial effect of the addition of extra B and extra Ca to the growth media. Compared with salt stressed nodules in media with normal B and Ca levels, the increase of the concentrations of both nutrients resulted in a recovery of the structure of the nodules. Moreover, salt-stressed nodules appeared devoid of rhizobia, and only the addition of both nutrients enhances

Therefore, Ca increases nodulation during growth of plants under salt stress and B is needed to enhance bacterial invasion and differentiation of N_2-fixing symbiosomes.

Table 6. Effect of different B (+B = 9.3 µM; +6B = 55.8 µM) and Ca^{2+} (+Ca = 0.68 mM; +4Ca = 1.36 mM) concentrations on nitrogen fixation (expressed as nmol C_2H_2 plant^{-1} h^{-1}), nodulation (nodules per plant), and growth of *Pisum sativum* plants grown in the presence of 75 mM NaCl 4 weeks post-inoculation with *Rhizobium leguminosarum*.

	Control (–NaCl)	+NaCl +B +Ca	+NaCl +B +4Ca	+NaCl +6B +Ca	+NaCl +6B +4Ca
Nitrogenase	217.4 ± 46.1	2.7 ± 1.9	1.8 ± 1.7	6.2 ± 2.4	138.2 ± 33.7
Nodules	67 ± 23	5 ± 3	38 ± 16	4 ± 4	46 ± 14
g (fw) shoot	2.3 ± 0.5	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	1.9 ± 0.5
g (fw) root	1.7 ± 0.4	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	1.6 ± 0.5

5.2.3. *Mineral composition of salt stressed plant grown under different B and Ca levels*

One of the effects of the addition of B and Ca on the increase of salt tolerance of nodulated pea can be due to the maintenance of the nutrient balance. As typically occurs, exposure of plants to salinity led to a massive entry of Na^+ and Cl^-, which is indicated by a high concentration of both ions in shoots and nodulated roots. Besides these toxic levels of Na^+ and Cl^-, one of the major constraints for plant growth on saline substrates is nutrient imbalance.

In nodulated pea, the measurement of B indicated that salinity provokes a deficiency of the micronutrient. Although Ca is able to recovers nutrient deficiency under salt stress (Cramer et al., 1987), it cannot recover the content of B in nodulated roots. Therefore, the increase of B is imperative. These measurements of nutrient content together with the importance of B and Ca in the development of the symbiosis justifies the alterations by high salt and the increase of salt tolerance in plants growing with a supplement of both nutrients.

Furthermore, not only B and Ca, but also some other nutrients (P, Mg, Mn, Cu . . .) are affected by high salt and recovered by B and Ca. Specially important for symbiotic N_2 fixation in legumes are potassium and iron. Potassium has a role in plant-water relations; it is the cation with a major contribution to the osmotic potential of cells in nonhalophytic plants (Hsiao and Läuchli, 1986). Functions of K^+ in higher plants include cell movements, cell extension, nutrient transport, cation-anion balance, and activation or stimulation of a large number of enzymes (see Marschner, 1995). Moreover, it has been shown that the symbiotic systems are more sensitive to low K than are the legumes themselves (Sangakkara et al., 1996), and a depression in the K^+ content at high salt levels is also typically detected. Therefore, the effects of K deficiency in these roots can be in part responsible for the low nitrogenase activity. The addition of Ca, especially at high B treatments, restored the amount of K^+ in nodulated plants.

Finally, salinity also reduced the concentration of Fe in nodulated roots, which is particularly recovered by 6B/4Ca treatments. A particular high requirement of iron exists in legumes not only for the nitrogenase complex, but also for the heme component of leghemoglobin and for the cytochrome oxidase of bacteroid electron transport chain (O'Hara et al., 1988). The iron content of roots of all of treatments

Table 7. Effect of different B (+B = 9.3 µM; +6B = 55.8 µM) and Ca^{2+} (+Ca = 0.68 mM; +4Ca = 1.36 mM) concentrations on the content of B (µg g^{-1} dry weight), Ca^{2+} (mg g^{-1} dry weight), K^+ (mg g^{-1} dry weight) and Fe (µg g^{-1} dry weight) in shoots and nodulated roots of *Pisum sativum* plants grown in the presence of 75 mM NaCl 4 weeks post-inoculation with *Rhizobium leguminosarum*.

	Control (–NaCl)	+NaCl +B +Ca	+NaCl +B +4Ca	+NaCl +6B +Ca	+NaCl +6B +4Ca
B in shoots	62 ± 8	23 ± 3	23 ± 2	71 ± 11	68 ± 5
B in roots	41 ± 4	16 ± 2	18 ± 2	25 ± 3	37 ± 4
Ca^{2+} in shoots	22 ± 5	7 ± 3	15 ± 3	11 ± 2	21 ± 3
Ca^{2+} in roots	8 ± 1	4 ± 1	7 ± 1	5 ± 1	9 ± 2
K^+ in shoots	30 ± 4	16 ± 3	22 ± 5	17 ± 4	25 ± 3
K^+ in roots	39 ± 9	18 ± 3	22 ± 3	22 ± 4	30 ± 6
Fe in shoots	180 ± 21	82 ± 12	70 ± 13	107 ± 22	169 ± 25
Fe in roots	172 ± 24	64 ± 13	61 ± 10	95 ± 23	215 ± 33

of salt-stressed plants is in the critical range of deficiency, 50–150 µg Fe g^{-1} dry weight, except nodulated roots of 6B/4Ca treatments.

Therefore, besides the recovery of nodule development by B and Ca, addition of both nutrients can prevent salt stress on nitrogen fixation in legume-rhizobia symbiosis by counteracting the effects of salt on nutrient balance.

In the system studied of *P. sativum* cv. Argona inoculated with *R. leguminosarum*, always a combination of 6 times increase of B and 4 times increase of Ca during plant growth was the best to increase salt tolerance. Other different treatments have only very small increases of tolerance or even inhibited plant and symbiosis development more than salt itself (i.e. B concentrations higher than 6 times normal were very toxic for plant growth). Consequently, as occur under physiological conditions, it might be an equilibrated nutritional status regarding B and Ca that produced the highest possible plant growth under salt stress. This status of equilibrium may change when the plant or the stressing-factor that affects plant nutrition changes and the use of a different B/Ca level should allow achieve it and again increase tolerance to the stress.

6. CONCLUDING REMARKS AND PERSPECTIVES

During the last two decades, a wide amount of studies demonstrating a role of boron for nitrogen fixation in free-living forms, both in cyanobacteria and bacteria actinomycete of the genus *Frankia*, and in legume or actinorhizal symbiosis have been developed, mainly in our group. These studies extended the role of the micronutrient not only to the process of biological nitrogen fixation, but also to the universe to be explorer of plant-microbe signalling. Boron deficiency can transform a symbiotic into a pathogenic relationship between a legume and its host rhizobia. Moreover, The role of Ca^{2+} in the signalling pathway in microbe and plants becomes every day more important. Therefore, the challenge of investigating the interaction between both nutrients during the dialogue of plants and microbes is simply amazing.

Nodulation and nitrogen fixation in legume-*Rhizobium* symbioses is dependent on boron (B) and calcium (Ca^{2+}). During early events of nodulation, B was essential for *nod* gene induction, root hair curling and adsorption of bacteria to root surface, though Ca^{2+} addition could prevent inhibitory effects of B deficiency and increased nodule number. High concentrations of Ca^{2+} also enhanced cell and tissue invasion by *Rhizobium*, which were highly impaired by B deficiency. Abnormal tissue differentiation of indeterminate (pea) and determinate (bean) nodules in the absence of B was restored by Ca^{2+} addition. Subsequently, the investigation of the B-Ca relationship on symbiosis has to be made at the molecular level. Our group, in collaboration with Drs. Adam and Eva Kondorosi (Institut des Sciences du Vegetal, CNRS, Gif-sur-Yvette, France), has initiated the study of the effects of B and Ca nutrition on gene expression during nodulation of the model legume *Medicago truncatula*. Macroarrays containing nodule cDNAs and RT-PCRs techniques showed an influence of B and Ca^{2+} concentrations on the level of expression of some genes implicated in nodule cell cycle regulation in B-deficient plants. Preliminary results on the analysis of key *cycD3* and *ccs52* genes (Foucher and Kondorosi, 2000) showed an overexpression of those genes in plants grown under B deficiency in early and late phases of nodule development, respectively. Besides, addition of Ca^{2+} cannot restore either the abnormal cell wall structure of B-deficient nodules or the distribution in the cell wall of pectin polysaccharides. Preliminary analysis of gene expression indicates that Ca^{2+} cannot also reduce overexpression of wall structural Hydroxyproline-Rich Glycoprotein in B-deficient nodules but diminished overexpression of wall loosening Expansin. Therefore, B and Ca^{2+} can play a complementary role in the establishment of the symbiosis, and both nutrients are essential for nodule structure and function, also by influencing expression of genes implicated in nodule development.

Finally, the study of symbiosis under salt stress indicates that a proper B and Ca nutrition can facilitate salt tolerance in the highly salt sensitive *Rhizobium*-legume N_2-fixing symbiosis. The addition of a Ca supplement can recover nodulation inhibited by salt, but a supply of B is also required for a correct nodule organogenesis and structure, which are damaged by salinity. Moreover, salinity also inhibits nitrogen fixation in nodules by the induction of deficiency of important nutrients as potassium and iron, which can be recovered by a balanced B and Ca nutrition. Such nutrition was 55.8 µM B and 2.72 mM Ca for *Pisum sativum* cv. Argona inoculated with *R. leguminosarum* strain 3841, but other pea cultivars and other legume species and genera would need a different optimal B/Ca ratio. Therefore, similar studies should accompany genetic approaches searching for tolerant cultivars, in order to establish the best B and Ca concentration for each type of legume that ensures the success of the symbiosis, plant development and crop production in saline soils.

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LIME-INDUCED IRON CHLOROSIS IN FRUIT TREES

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1. INTRODUCTION

Iron deficiency (iron chlorosis) is an important nutritional disorder in fruit trees that results not from a low level of iron in soils but from impaired acquisition and use of the metal by plants. Calcium carbonate, present in great amounts in the same soils, and the resulting high level of bicarbonate ions, are the main causes of iron deficiency.

Countries in southern Europe, such as Portugal, Spain, Italy and Greece, have large areas of calcareous soils with established orchards, where iron chlorosis is a major factor that limits yield and profit for the farmer.

Iron chlorosis affects several metabolic processes and leads to nutrient imbalances in the plant. Decreased yield and poor quality of fruits resulting from the deficiency justify the development of methods to diagnose and correct this disorder. No single approach has been found to solve iron chlorosis satisfactory, making it one of the most complex nutritional deficiencies.

In this chapter we describe the main aspects of iron nutrition in calcareous soils and plant response mechanisms to iron deficiency, and then concentrate on reviewing current methods to detect and correct iron chlorosis in fruit trees.

1.1. Iron in soils

Iron is the fourth most abundant element in the lithosphere, after oxygen, silicon and aluminium. In primary minerals iron is mainly in the ferrous form, as part of the structure of ferromagnesium silicates such as biotite, olivine, augite, and hornblende. These minerals weather by oxidation and hydrolysis releasing iron that may be precipitated under aerobic environments as oxides, oxyhydroxides or carbonates of Fe (III).

The most abundant Fe-containing secondary mineral is haematite (α-Fe_2O_3) due to its great thermodynamic stability (Krauskopf, 1983). Other oxides and oxyhydroxides of iron are maghaemite (γ-Fe_2O_3), magnetite (Fe_3O_4), ferrihydrite ($Fe_2O_3.nH_2O$) and goethite (α-FeOOH). Only a small percentage of the iron released by weathering is adsorbed onto clay minerals or organic matter (Lindsay, 1991, 1995; Loeppert, 1986).

The iron content of soils varies from 0.02% in sandy soils to more than 10%

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in Ferrasols, with an average of about 3.8% (Chen and Barak, 1982). However, in most soils the concentration of ionic iron (Fe^{3+} and Fe^{2+}) in solution is very small, usually less than 10^{-15} M (Marschner, 1995). Iron forms stable complexes with organic ligands, such as citrate, and in consequence chelates of Fe (III) and occasionally Fe (II) are the major species in soil solution (Marschner, 1995).

The activity of iron in soil solution depends on the relative solubility of the different species present, controlled by characteristics of the solid phase (type of mineral, specific area, degree of cristalinity and organic matter content) and the liquid phase (pH, redox potential, and concentration of reactants) (Schwertmann, 1991). At the root surface and in the rhizosphere the mobility of iron may be distinctly different from that in bulk soil due to microbial activity and differential uptake by plants of cations and anions that modify pH and redox potential (Marschner, 1991; Römheld and Marschner, 1986b). Microorganisms can create small anaerobic pockets and release siderophores (Uren, 1993), which chelate iron and increase its bioavailability (Masalha et al., 2000). These mechanisms are especially important when iron in solution is scarce, such as in calcareous soils (Marschner, 1991).

1.2. Iron in plants

Iron cannot be considered a trace element in soils, but its requirement to plants is sufficiently small for the metal to be classified as a micronutrient. Iron plays essential roles in several biochemical processes due to its affinity for many organic ligands and its capacity to change the oxidation state from (II) to (III).

1.2.1. *Uptake mechanisms*

Though in well-aerated soils oxidised forms of iron prevail, dicots can only absorb Fe^{2+} (Chaney et al., 1972; Wang and Peverly, 1999). The absorption of iron thus begins with its reduction by a plasmalemma-bound 'standard reductase' that transfers electrons from cytosolic reductants to the apoplast (Brüggemann et al., 1990; Buckhout et al., 1989; Grusak et al., 1999; Holden et al., 1992; Holden et al., 1991; Rubinstein and Luster, 1993; Schmidt, 1999). It is still unclear whether the enzyme uses nicotinamide-adenine-dinucleotide (NADH) or nicotinamide-adenine-dinucleotide phosphate (NADPH) as the electron source (Moog and Brüggemann, 1994; Schmidt, 1994; Schmidt and Bartels, 1998; Schmidt and Janiesch, 1991; Schmidt and Schuck, 1996). This standard reductase, a constitutive enzymatic system, is always present in root apices, both in dicots and monocots, whatever the level of iron in the soil. The enzyme has affinity for substrats with low redox potential (e.g. ferricyanide), but is involved in processes other than iron uptake, such as membrane polarisation, and the control of cell elongation and division (Moog and Brüggemann, 1994; Schmidt, 1999; Welch, 1995). We will consider the reductase that is induced under iron deficiency in section 2.2.3.

Iron reduction in intact roots takes place under relatively acid conditions, at around pH 5 (Susín et al., 1996a, b), while by using detached plasmalemma vesicles the optimum pH obtained was 6.8 for barley (Brüggemann et al., 1993) and 6.5 for tomato (Holden et al., 1991). However, this difference may be due to the rupture

of some membranes and the contamination of the preparation with cytosol (Abadía, 1998; González-Vallejo et al., 1999).

Siderophores (of plant or microbial origin) chelated to Fe (III) and synthetic Fe-chelates can also be absorbed, albeit in smaller amounts that ionic iron, via the apoplast (Marschner et al., 1988). This process occurs preferentially in basal zones of the roots, where lateral branches emerge (Marschner, 1991; Marschner et al., 1987). Consequently, soluble iron compounds in the apoplasm of the cortex, ionic iron adsorbed to cell wall exchange sites, or even freshly precipitated amorphous Fe(OH)$_3$, can also function as sources of iron for plant uptake (Bienfait et al., 1985; Zhang et al., 1999).

After uptake into rhizodermal cells, iron moves across the cortical cells towards the xylem vessels (Figure 1). During this radial movement, the element is probably chelated by nicotianamine (Higuchi et al., 1995; Stephan et al., 1995; Stephan and Scholz, 1993) to avoid adsorption to cell walls and the oxidative damage that would result from the production of oxygen and hydroxyl free radicals by Fe^{2+} (Grusak et al., 1999; Welch, 1995).
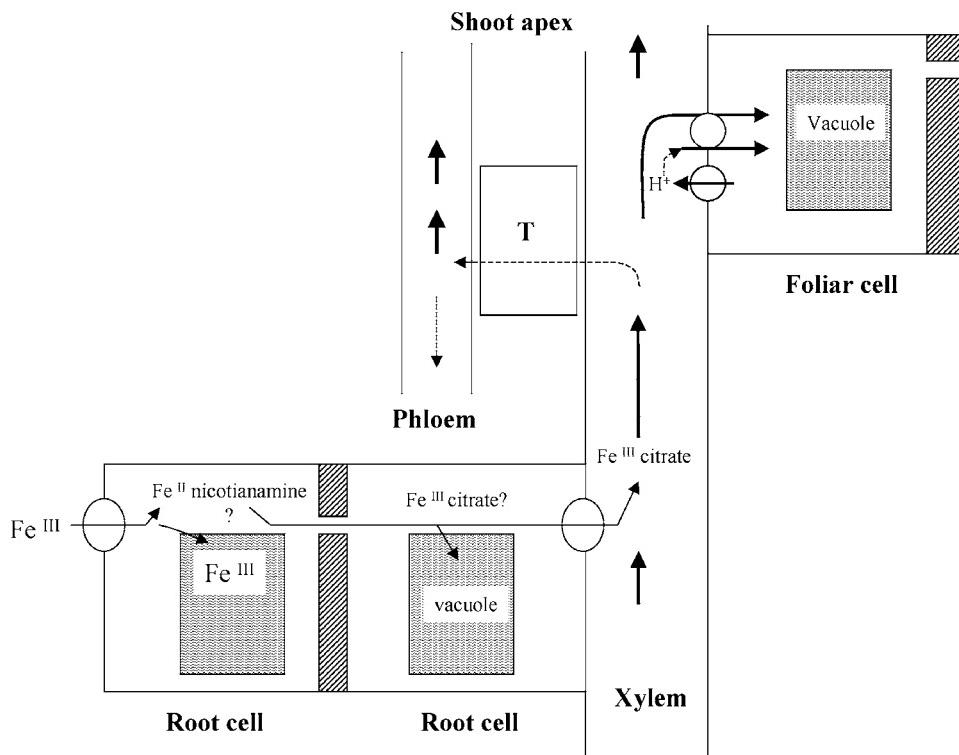
Figure 1. Possible model for the radial transport of iron in root symplasm and for long distance transport to the shoot. The xylem to phloem transfer of iron and its distribution in leaves are also represented. T – transfer cell, ⊖→ – reductases. Question marks refer to steps that are poorly characterised. Adapted from Marschner (1991).

1.2.2. *Translocation to shoots*

Prior to xylem loading, the Fe (II) chelated by nicotianamine is re-oxidised and chelated as Fe (III)-citrate, which seems to be the main compound involved in iron transport in the xylem (Brown and Jolley, 1989). However, Rombolà et al. (2000) considered that citric acid is used as the chelator only when other organic acids are present in xylem vessels at similar concentrations. When other organic acids predominate, for example malic acid is detected in high concentrations in the xylem of several plant species, they may substitute for citric acid in iron chelation (Bialczyk and Lechowski, 1992; Clark and Zeto, 2000; López-Millán et al., 2000a; López-Millán et al., 2000b; Rombolà et al., 1998a).

The driving forces for the transport of Fe-chelates in the xylem vessels are transpiration and root pressure (Marschner, 1991). Transpiration regulates transport into fully expanded leaves, with small demand for the element, while root pressure mediates transport to the sites of great demand, including the growing parts such as shoot apices, expanding leaves, and developing fruits and seeds. Transport due to root pressure is confined to periods of low transpiration, such as during the night (Marschner, 1991; Welch, 1995).

Iron supply to meristems, especially shoot apices, can occur via xylem or phloem (Grusak et al., 1999). Kosegarten et al. (1999) showed that during the early stages of leaf development in sunflower (leaves up to about 800 mm^2) the main source of iron was Fe (III)-citrate carried in xylem vessels. Conversely, in iron-deficient plants, the remobilisation of iron reserves from leaves must involve phloem transport. Iron can also transfer from xylem into phloem during the upward transport, a process that is probably mediated by highly specialised cells, called transfer cells (Landsberg, 1984) (Figure 1). According to Grusak et al. (1999) for successful xylem-to-phloem exchange, iron must cross the membrane surrounding the phloem sieve-tube companion cells and then move in the symplasm (via plasmodesmata) towards the sieve tubes. Nicotianamine seems to be a phytometallophore essential for phloem transport of the metal (Krügger et al., 2002; Welch, 1995).

1.2.3. *Functions of iron in plant metabolism*

After translocation to shoots, the uptake of iron into leaf mesophyll cells depends on the reduction of ferric citrate carried out by a plasmalemma-bound Fe (III)-chelate reductase (FC-R), first identified by Brüggemann et al. (1993) in *Vigna unguiculata*. This leaf reductase is somewhat similar to the redox system in roots since it also depends on plant metabolic activity and apoplastic pH. In intact leaves, the maximum rate of iron reduction was detected at an apoplastic pH of 5.0, in accordance with the cell wall buffer capacity ($pK_a = 5$) (Kosegarten et al., 1999).

The FC-R has the capacity to reduce Fe (III) either from citrate or malate (Larbi et al., 2001; Rombolà et al., 1998a), consistent with the hypothesis that not only citric acid but also other organic acids can chelate iron prior to translocation to shoots.

In leaves, reduction of Fe-chelates is stimulated by light, associated with an increase in the $NAD(P)H:NAD(P)^+$ ratio through photosynthesis (Brüggemann et al.,

1993; Nikolic and Römheld, 1999; Rubinstein and Luster, 1993). This photoreduction of Fe (III) may play a significant role in iron uptake by mesophyll cells (Alcántara et al., 1994; Larbi et al., 2001; Pushnik and Miller, 1989; Schmidt, 1999; Welch, 1995).

Once in the cytoplasm, Fe^{2+} is probably chelated by nicotianamine and then distributed for use in the different metabolic processes where iron participates (Scholz et al., 1992; Stephan and Scholz, 1993). Data available suggests that entry into chloroplasts also involves an active transport (Abadía, 1998; Terry and Low, 1982).

Iron can change its oxidation state in biological systems between (II) and (III) and forms stable octahedral complexes with various ligands that result in different redox potentials. In higher plants, iron is essential in several metabolic processes such as photosynthesis, respiration, N_2 fixation, and nitrate reduction (Welch, 1995).

Iron is incorporated into haeme or nonhaeme proteins (Miller et al., 1995). Examples of haeme proteins, which represent about 9% of total foliar iron, are cytochromes, nitrogenase, leghaemoglobin, catalase and several peroxidases. Well-known nonhaeme proteins are ferredoxin, aconitase and xanthine oxidase. Together these nonhaeme proteins constitute about 19% of foliar iron. Cytochromes are components of the redox systems in chloroplasts and mitochondria. Nitrogenase and leghaemoglobin are essential for the biological nitrogen fixation that takes place in the nodules of legumes. Catalase, in association with superoxide dismutase, assists the dismutation of H_2O_2 to water and O_2. Peroxidases catalyse the polymerisation of phenols to lignin (Marschner, 1995; Nenova and Stoyanov, 1995). Ferredoxin contains an iron-sulphur cluster and acts as an electron donor in several metabolic processes such as photosynthesis and nitrate reduction (Miller et al., 1984; Smith, 1984). Iron, as part of the prosthetic group, is required for the stability and activity of aconitase, another iron-sulphur protein. Aconitase catalyses the isomerisation of citrate to isocitrate in the tricarboxylic acid (Krebs) cycle (Smith, 1984). Xanthine oxidase participates in purine metabolism.

The metabolic pathways for the synthesis of the porphyrin structure of chlorophyll, cytochromes and haeme proteins are very similar. Although iron is not present in the chlorophyll molecule, it controls the rate of δ-aminolevulinic acid (ALA) synthesis and is also required for the formation of protochlorophyllide from Mg-protoporphyrin (Miller et al., 1995; Pushnik et al., 1984). Iron is also essential in the synthesis of proteins required for the development of the lamellae structure of chloroplasts (Abadía et al., 1989a).

Iron cannot be present as a free ion in cells because it would lead to oxidative damage. The reserves of the metal accumulate in the form of phytoferritin particles in the stroma of plastids (about 35% of total foliar iron) (Abadía, 1992, 1998; Briat et al., 1995; Macur et al., 1991; Smith, 1984).

2. THE DEVELOPMENT OF IRON CHLOROSIS

Iron deficiency results in a decrease in the concentration of photosynthetic pigments in leaves, usually referred to as iron chlorosis (Abadía, 1992; Abadía and Abadía,

1993; Terry and Abadía, 1986). There is no significant remobilisation of the metal when uptake does not meet demand due to the small mobility of iron in the phloem. Hence, the symptoms of the deficiency occur primarily in young leaves and became apparent as an interveinal chlorosis with the appearance of a fine reticulation.

Iron chlorosis can result from insufficient supply in organic soils, in coarse-textured leached soils, or in intensively cultivated soils. Most commonly, however, iron chlorosis is a consequence of factors that interfere with the availability, acquisition or utilisation of iron by plants. The identification of these factors and of their effects on metabolic pathways in the plant can help to establish methods to diagnose and correct this important nutritional disorder.

2.1. Factors that induce iron chlorosis

Iron chlorosis can be induced by factors that affect the activity, integrity and length of the root system, such as low or high soil temperatures (Wei et al., 1994; Welkie, 1995), soil compaction, poor aeration (Loeppert, 1986), and root damage by tillage, nematodes and other organisms (Brown, 1961). The deficiency may also be due to factors that decrease the level of iron in soil solution, such as high redox potential (Chaney et al., 1989; Kolesh et al., 1987a, b; McCray and Matocha, 1992), small organic matter content (Lucena, 2000), and alkalinity, for example that resulting from bicarbonate in soil or irrigation water (Loeppert et al., 1988).

Iron chlorosis can also be induced or enhanced by other nutrients, such as nitrogen, magnesium, phosphorus, calcium, manganese, zinc and copper (Wallace et al., 1992). Nitrogen may cause or alleviate iron chlorosis, depending on the form supplied. Nitrate can induce iron chlorosis because the ion crosses the plasma membrane by a proton-anion (H^+/NO_3^-) cotransport, increasing rhizosphere and apoplastic pH (Kosegarten and Englisch, 1994; Kosegarten et al., 1999; Lucena, 2000). Ammonium has the reverse effect since as a cation its uptake leads to decreased rhizosphere pH and therefore enhances iron uptake (Hoffmann et al., 1992). Similarly, several authors refer the role of potassium in reducing iron chlorosis due to its effect on rhizosphere acidification (Hughes et al., 1990, 1992; Jolley et al., 1992; Wallace et al., 1992).

Several studies (e.g. Mengel et al., 1984; Wallace et al., 1992) confirm that an interaction between phosphorus and iron takes place, both in soils and plants, especially in calcareous soils (Aktas and Van Egmond, 1979). Precipitation of ferric phosphate was reported in roots of tomato plants (Ayed, 1970).

High levels of other micronutrients (manganese, copper and zinc) may impair iron nutrition. The metals compete with iron for ligands both in soils and plants (Mengel et al., 1984; Natt, 1992; Wallace and Wallace, 1992). Manganese can substitute for iron in catalase and peroxidase, as shown in citrus (Lavon and Goldschmidt, 1999; Thomas et al., 1998; Zaharieva, 1995). Depending on their concentration, zinc and copper can competitively inhibit access of iron to chelators, thereby decreasing iron uptake from soil (Alva and Chen, 1995; Jolley and Brown, 1994; Schmidt et al., 1997), although the activity of copper in calcareous soils is usually very small because it is complexed by organic substances (Lindsay and Schwab, 1982).

Some non-essential elements, such as chromium, cadmium and aluminium, can also induce or enhance iron chlorosis (Schmidt et al., 1996; Siedlecka and Krupa, 1999). Aluminium toxicity, frequent in acid soils, and cadmium can induce iron chlorosis because they inhibit the biosynthesis and secretion of phytosiderophores by graminaceous species (e.g. wheat and sorghum) (Brown and Jolley, 1989; Chang et al., 1998).

The most prevalent cause of iron chlorosis in the Mediterranean area is the bicarbonate ion, which occurs in high levels in calcareous soils. It is estimated that from 20 to 50% of fruit trees in the Mediterranean basin suffer from iron chlorosis (Jaegger et al., 2000). Calcareous soils often have more than 20% of calcium and magnesium carbonates; consequently, they are strongly buffered, with a pH between 7.5 and 8.5. The relatively small precipitation (<500 mm), typical of these regions with arid and semi-arid climates, enhances iron chlorosis (Loeppert, 1986). Drought stress can result in increased abscisic acid (ABA) concentrations leading to a rise in pH of up to 2 units in the xylem and leaf apoplast. The consequence is then an inhibition of leaf growth. The release of phytosiderophores by roots is also affected (Römheld and Awad, 2000).

Under oxidising soil conditions, soluble ferric and ferrous salts react rapidly with calcium carbonate to form solid Fe-hydroxides as represented in the following reactions (Loeppert, 1986):

$$4Fe^{2+} + O_2 + 4CaCO_3 + 2H_2O \rightarrow 4FeOOH + 4Ca^{2+} + 4CO_2 \qquad (1)$$

$$2Fe^{3+} + 3CaCO_3 + 3H_2O \rightarrow 2Fe(OH)_3 + 3Ca^{2+} + 3CO_2 \qquad (2)$$
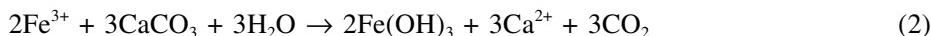
The compound formed depends on the reactive surface area of calcium carbonate, and on the partial pressures of O_2 and CO_2. At pH lower than 7.4, ferrihydrite ($Fe_2O_3.nH_2O$) is the dominant form; between pH 7.4 and 8.5 goethite (FeOOH) is (Eq. 1), and at pH higher than 8.5 ferric hydroxides ($Fe(OH)_3$) are formed (Eq. 2) (Lindsay, 1995; Schwertmann, 1991). According to Lindsay and Schwab (1982), for each increment of one unit in pH the ionic iron solubility drops a thousand times. Within the pH range of most calcareous soils the concentration of dissolved iron is approximately 10^{-10} M, considerably less than the range of values (10^{-4} to 10^{-8} M) required for optimum plant growth (Haleem et al., 1995; Lindsay, 1991; Welch, 1995).

The concentration of bicarbonate ions in the soil solution of calcareous soils, resulting from the dissolution of calcium carbonate, can be over 200 g HCO_3^- kg^{-1} in some circumstances, depending on the partial pressure of CO_2 (Loeppert, 1986). Bicarbonate can be continuously formed at root surfaces, where respiration provides CO_2 for the dissolution of calcium carbonate (Mengel, 1995). After bicarbonate, nitrate is the second main anion that induces iron chlorosis in calcareous soils (Bar and Kafkafi, 1992; Kosegarten and Englisch, 1994; Kosegarten et al., 1999; Kosegarten et al., 1998b; Smolders et al., 1997). In these soils, NO_3^- is the main form of mineral nitrogen in soil solution due to intense nitrification and NH_3 volatilisation (Kosegarten et al., 1999). Uptake of nitrate by plants contributes to rhizosphere alkalinity.

2.2. Root response mechanisms to iron deficiency

Roots have mechanisms that promote the solubility and availability of iron in the rhizosphere, which can be classified as non-specific ('constitutive-' or 'standard-system') and specific ('inducible-system') (Römheld, 1987a, b). Non-specific mechanisms are always present in plants, irrespective of their nutritional status. In contrast, specific mechanisms are activated when the iron concentration in plant tissues decreases below a critical level, and are disabled at an optimum threshold and before toxic levels are reached.

Examples of non-specific mechanisms are i) rhizosphere acidification caused by preferential cationic (K^+ and NH_4^+) absorption by roots (Mengel, 1995; Römheld et al., 1984); ii) release of organic compounds, which can protect roots or enhance iron complexation (Marschner et al., 1986; Masalha et al., 2000); and iii) root cation exchange capacity (CEC) that results in iron adsorption to binding sites in cell walls (Bakker and Nys, 1999). Genotypic differences between calcicole and calcifuge species are closely related to the CEC, with many calcicoles being able to tolerate high levels of soluble calcium, or to sequester it as insoluble calcium oxalate in cell vacuoles (Kerley, 2000a, b). Hamzé et al. (1980) observed that tolerant citrus rootstocks had greater CEC than susceptible genotypes, an intrinsic characteristic not induced by iron chlorosis.

Microbial activity can also increase iron absorption due to the release of several ligands such as organic acids, sugars and siderophores, and to the development of anaerobic microsites that favour iron reduction (Awad et al., 1995b; Cress et al., 1986; Jurkevitch et al., 1992; Lindsay, 1991). The beneficial effect of the symbiotic relationship between legumes and rhizobia is related to siderophore release (Bar-Ness et al., 1991; Walter et al., 1994). Mycorrhizas also improve iron nutrition due to an increased surface area (roots plus fungi) for nutrient acquisition (Clark and Zeto, 2000; Marschner, 1998). In contrast, microbial root colonization can impair iron nutrition due to competition for photosynthates and nutrients between host and microbe (Marschner, 1998; Marschner et al., 1986). In citrus seedlings, the favourable effects of microbial root colonization seemed to be limited to acid conditions in the soil environment (Treeby, 1992).

Higher plants have distinctive behaviours when faced with iron chlorosis so that they can be segregated into two groups: efficient and non-efficient plants. Efficient species are further separated phylogenetically into two groups: those following Strategy I, and those that adopt Strategy II (Marschner et al., 1986). Strategy I is found in dicot and monocot species, with the exception of members of the *Poaceae* (*Gramineae*) families. Strategy II is confined to grasses.

Strategy II-plants rely on the secretion of phytosiderophores into the rhizosphere together with the induction of a high-affinity system for Fe (III)-phytosiderophore uptake (Gahoonia et al., 2000; Gerke, 2000; Ohata et al., 1993; Römheld, 1987a, b; Römheld and Marschner, 1986b, 1990; Scholz et al., 1992; Singh et al., 2000; Yehuda et al., 1996). Strategy I-plants have several mechanisms to increase iron uptake, which include proton extrusion, secretion of chelators, enhanced Fe (III) reduction, and increased activity of Fe^{2+} transporters in the root plasmalemma (Bienfait et al., 1985; Grusak et al., 1999; Jolley and Brown, 1994; Römheld, 1987b;

Römheld and Awad, 2000; Römheld and Marschner, 1986a, b, 1990; Schmidt, 1999; Welch, 1995). Since fruit trees belong to the Strategy I group, we will describe their response in more detail.

2.2.1. *Rhizosphere acidification*

Iron deficiency promotes proton extrusion in Strategy I-plants, resulting in rhizosphere acidification, a process mediated by H^+-ATPases located in root plasma membranes (Serrano, 1989; Susín et al., 1994; Vos et al., 1986; Welkie, 1993). In subterranean clover, Wei et al. (1998) showed that a critical level of iron in the plant triggered the increased acidification.

2.2.2. *Release of reductants and chelators*

Secretion of phenolic compounds and flavines, and the accumulation of organic acids and polypeptides in roots and shoots, is another consequence of iron deficiency in Strategy I-plants (Bienfait et al., 1983; Buckhout et al., 1989; Marschner et al., 1986; Römheld, 1987a, b; Vempati et al., 1995). Phenol secretion may result from the lack of incorporation of these compounds into suberin, probably as a result of decreased extracellular suberin peroxidase activity in iron-stressed root tips (Sijmons and Bienfait, 1984; Welkie, 1993). The phenolic compounds most frequently detected are caffeic and chlorogenic acids, which may act as chelators for Fe (III) (Alhendawi et al., 1997; Römheld and Kramer, 1983).

Secretion of flavines was detected in peppers (Welkie, 1993) and sugar beet (Susín et al., 1994; Susín et al., 1993). Susín et al. (1994) noted that the release of flavines only occurs with rhizosphere acidification. In alkaline conditions these compounds can accumulate to 1 mM in roots. The function of these compounds in root metabolism has yet to be fully elucidated. However, they play a role in the reduction of ferric compounds in the presence of NAD(P)H, and interact with the Fe (III)-chelate reductase (González-Vallejo et al., 1998a; González-Vallejo et al., 1998b). Several authors (Alhendawi et al., 1997; Fournier et al., 1992; Gerke et al., 1994; López-Millán et al., 2000b; Rombolà et al., 1998a) reported the accumulation of various organic anions, especially malate and citrate, in roots and shoots of plants with iron chlorosis. Organic acid accumulation may result from increased phosphoenolpyruvate carboxylase (PEPC) activity (Abadía, 1998; Andaluz et al., 2000; González-Vallejo et al., 1998b; Landsberg, 1984; López-Millán et al., 1998; López-Millán et al., 2000b; Rabotti and Zocchi, 1994; Suzuki et al., 1995). Organic acids favour iron reduction and translocation to shoots and regulate cellular pH (Marschner et al., 1986). In an assay with iron-deficient sugar beet roots, López-Millán et al. (2000a) reported that flavines could act as a metabolic link between organic acids and Fe (III)-chelate reductase.

2.2.3. *Enhanced reduction of Fe (III)*

Strategy I-plants are also capable of enhancing the reduction of iron linked in Fe (III)-chelates by an inducible ('turbo') reductase localised on the plasma membrane

of rhizodermal cells. This enzyme differs from the standard system covered in detail in section 1.2.1. The model with two different reductase activities was first proposed by Bienfait et al. (1985) and later confirmed by several authors (Brüggemann et al., 1990; Moog and Brüggemann, 1994; Romera et al., 1991c; Römheld, 1987a, b).

The molecule carrying reductive potential that function in conjunction with the turbo reductase has not been identified yet. Moog and Brüggemann (1994) found that NADH was the electron donor when the reductase was assayed *in vitro*, but Sijmons et al. (1984) showed it to be dependent on NADPH but not NADH, when using bean roots *in vivo*.

The rate of Fe (III) reduction is maximal at around pH 5.5 *in vivo* and pH 7.0 *in vitro* (Grusak et al., 1999; Moog and Brüggemann, 1994; Schmidt, 1999). The reductase seems to interact with the plasma membrane components as part of an electron transport system (Bagnaresi and Pupillo, 1995; Schmidt et al., 1996; Susín et al., 1996a, b). Robinson et al. (1999) identified one flavoprotein that was needed for its activity in *Arabidopsis*.

Although some progress in the biochemical characterization of the enzyme has taken place, it is still not clear if the enhanced reductive capacity results from activation of an existing reductase, or if there is an induction of a novel protein. The available data point to an increased expression of an enzyme distinct from the standard reductase (for more complete reviews see Grusak et al., 1999; Schmidt, 1999).

Contrary to the observation of Susín et al. (1994) in sugar beet, the increase in the reductase depends on the presence of small amounts of iron in solution in several plant species that include beans (Chaney et al., 1972), soybean (Tipton and Thowsen, 1985), sunflower ((Romera et al., 1992), peas (Grusak et al., 1993), tomato (Zouari et al., 2001), orange (Pestana et al., 2001c), and peach (Gogorcena et al., 1998, 2000). The requirement for a small amount of iron may be due to its effect on the activity of the 1-aminocyclopropane-1-carboxilic acid (ACC) synthase. This enzyme plays a role in ethylene biosynthesis, a putative regulator of Fe-deficiency responses in plants. However, in orange plants a low level of iron was not sufficient by itself to induce an increase in the activity of the Fe (III)-chelate reductase, and the presence of calcium carbonate was also required, suggesting that the regulation was also dependent on pH (Pestana et al., 2001c).

An increase in the activity of the reductase related to iron chlorosis has been shown to occur in several fruit crops such as grape (Bavaresco et al., 1991; Brancadoro et al., 1995; Dell'Orto et al., 2000), apple (Ao et al., 1985), peach (Cinelli et al., 1995; de la Guardia et al., 1995; Egilla et al., 1994; Gogorcena et al., 1998, 2000; Romera et al., 1991a, b), quince (Cinelli, 1995; Tagliavini et al., 1995a; Viti and Cinelli, 1993), pear (Tagliavini et al., 1995b), kiwi (Vizzotto et al., 1997; Vizzotto et al., 1999), and citrus (Manthey et al., 1993, 1994; Pestana et al., 2001c; Treeby and Uren, 1993). However, in iron deficient plants of peach (Romera et al., 1991b), pear and quince (Tagliavini et al., 1995b) enzymatic activity of FC-R was less than in Fe-sufficient plants probably due to the different methodology used as discussed by Gogorcena et al. (2000).

The location of the inducible enzymatic system seems to vary among species (Grusak et al., 1999). In some the reduction activity is restricted to sub-apical root zones (Chaney et al., 1992; Marschner et al., 1986; Römheld and Marschner, 1986a), while in others this process occurs in the whole root (Grusak et al., 1993). Sometimes, the enhancement in iron reduction is even confined to root hairs (Römheld, 1987a, b).

2.2.4. *Morphological root changes*

Physiological adaptations to iron deficiency may be associated with morphological changes such as subapical swelling of roots, formation of new root tips extending from the swollen zones, and formation of root hairs and transfer cells (Egilla et al., 1994; Landsberg, 1995; Romera and Alcántara, 1994; Römheld and Marschner, 1979, 1981; Welkie, 1993). However, Schikora and Schmidt (2001) stated that morphological changes seem to be induced only when physiological mechanisms cannot overcome the deficiency and provide adequate levels of iron.

Rhizodermal transfer cells are characterized by cell-wall ingrowths, relatively small vacuoles, dense cytoplasm, and abundant mitochondria (Landsberg, 1984, 1995). The ingrowths of secondary wall material lead to an enlargement of the plasma membrane (up to 20-fold) and consequently, a great number of proton-pumps and electron-exporting sites (Kramer et al., 1980; Schmidt and Bartels, 1996; Welkie and Miller, 1993). Furthermore, the abundance of mitochondria may generate the extra energy required for processes induced under Fe-deficiency (Schmidt and Bartels, 1996).

The structure of transfer cells differs between species, ranging from little-modified cells in *Plantago* (Schmidt and Bartels, 1996) to complex labyrinth-like cells in Fe-stressed roots of *Capsicum* (Landsberg, 1995) and *Helianthus* (Kramer et al., 1980). Schmidt and Bartels (1996) even proposed the classification of species in three groups based on these characteristics (labyrinth, papillary or small wall ingrowths) and on the number of transfer cells induced by iron deficiency (more or less than 50% of rhizodermal cells).

2.2.5. *Regulation and efficiency of root responses*

Shoot-to-root communication seems to take place and up-regulate a number of specific nutrient-dependent mechanisms. The nature of the signal in Fe-deficient plants has not been determined, although plant hormones, Fe-binding compounds and even re-translocated iron have all been suggested as possible messages used to mediate and regulate Fe-deficiency responses in roots (Bienfait et al., 1983; Grusak and Pezeshgi, 1996; Landsberg, 1984; Römheld and Marschner, 1986a; Rubinstein and Luster, 1993; Schmidt, 1999; Schmidt et al., 2000).

Landsberg (1995) studied the alterations in endogenous hormonal balances associated with the induction of root responses to iron deficiency. In view of the fact that exogenous indole-3-acetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) can also reproduce some of the morphological changes induced by iron chlorosis,

it has been postulated that auxins are associated with these processes (Schmidt, 1999). However, Romera et al. (1996; 1999) proposed ACC as another possible regulator of the iron deficiency response in plants. Schmidt (1999) produced a working model that included multiple hormonal effects on iron deficiency responses to take into account the information obtained by several authors. Recently, Li et al. (2000) working with cucumber and beans concluded that the shoot plays an important role in the regulation of the root reductase in Fe-deficient plants, but there were differences between the two plant species in the signal molecule.

The responses to Fe-deficiency point to a feedback mechanism, since after leaf re-greening the various processes are deactivated (López-Millán et al., 2001a; Marschner et al., 1986; Schmidt et al., 1996).

The presence of a response mechanism in plants is not necessarily associated with tolerance to lime-induced chlorosis, since this depends on the amplitude of physiological changes that occur in calcareous soils (Römheld and Marschner, 1986b). In addition to the mechanisms already described, some Fe-tolerant vine rootstocks decreased their growth rate and enhanced the iron use efficiency (Bavaresco et al., 1994). Other mechanisms related to tolerance present in subclovers (*Trifolium* sp.) include (Wei et al., 1995) i) larger root:shoot ratios under iron deficiency; ii) more balanced nutrition; iii) more effective mobilization of soil iron; and iv) smaller requirement for iron in shoots, corresponding to a greater use efficiency. In Fe-deficient peach trees, the rootstock tolerance was related to the capacity to develop a root area large enough to secure appropriate iron reduction and absorption (Egilla et al., 1994). Cultivars of apple that are more tolerant to iron chlorosis have greater cell wall cation exchange capacity, electrical conductivity, and capacity to lower rhizosphere pH to favourable values compared with susceptible cultivars (Han et al., 1998).

2.3. Effects of iron chlorosis on shoots

Lime-induced iron chlorosis affects the translocation of iron from roots to shoots and its distribution within leaves (Grusak et al., 1999; Loeppert, 1986; Marschner, 1991; Mengel, 1995; Mengel et al., 1994). There is an accumulation of inactive iron in the apoplast of leaves (González-Vallejo et al., 2000; Kosegarten et al., 1999; Morales et al., 1998c) related to impaired xylem unloading and cell uptake (Stephan and Scholz, 1993).

It has been reported that the effects of iron chlorosis on long distance transport of iron were due to an increase in the sap pH as a result of bicarbonate ions (Mengel et al., 1994). However, some authors claim that sap pH actually decreases after bicarbonate addition to nutrient solutions (Bialczyk and Lechowski, 1992). Lucena (2000) stated that the total amount of iron in the xylem is small, and not much affected by the level of bicarbonate in the growth medium. It seems clear that further evaluation of the factors that affect transport of iron to shoots has to be carried out, and these studies have to bear in mind that iron may be complexed by several organic acids as discussed in section 1.2.2.

2.3.1. *Iron mobility in leaves*

Once in the leaf apoplast, iron has to cross the plasma membrane to be used by leaf cells. Iron reduction takes place before uptake, a process mediated by a plasmalemma-bound Fe (III)-chelate reductase (FC-R) with an optimum pH of around 5.0. Consequently, the leaf FC-R plays an important role in the uptake of iron by mesophyll cells, and in the physiological availability of the nutrient in the plant. Under alkaline conditions Fe (III) reduction is depressed, inducing leaf chlorosis (Kosegarten et al., 1999).

Mengel (1994) suggested that a large bicarbonate concentration in the soil could result in an increase in the pH of the leaf apoplast, but Nikolic and Römheld (1999) have recently observed that the rise in apoplast pH was independent of both the nutritional status of iron and the presence of bicarbonate ions in the plant. Furthermore, the accumulation of bicarbonate ions in the shoots is very unlikely since after absorption by root cells this anion is converted into organic acids, like malic acid, and it is this form that is translocated to the shoots (Bialczyk and Lechowski, 1992). According to Kosegarten et al. (1999) the high pH of the apoplast may be a consequence of a nitrate-based nutrition, the nitrogen form prevalent in calcareous soils.

A few authors do not link iron chlorosis with an increase in apoplastic pH. López-Millán et al. (2000a, 2001b) even reported that iron deficiency caused a slight decrease in the pH of the leaf apoplast (from 6.3 to 5.9) and xylem sap (from 6.0 to 5.7) in sugar beet, while in leaves of pear trees the apoplastic pH increased almost one unit under iron chlorosis but did not seem to be the main cause for poor iron acquisition by mesophyll cells. This was explained by changes in the ratios of cations and anions in the apoplastic sap (López-Millán et al., 2001b). According to these authors the increase in the concentration of organic anions in leaves, which results from absorption of bicarbonate ions, may even improve the activity of the leaf reductase. Iron chlorosis could then result from changes in leaf metabolism such as increases in Krebs cycle enzymatic activities, pyridine pools, and in the ratio NAD(P)H:NAD(P)$^+$. In spite of these contradictory results, the fact is that most authors observe a decrease in the activity of the leaf reductase in chlorotic plants. In contrast to what is observed in roots, no induction of plasmalemma-bound reductase has been identified in leaves (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; Larbi et al., 2001; Nikolic and Römheld, 1999; Rombolà et al., 2000). Instead of this, the development of transfer cells around xylem vessels, observed in sunflower plants under iron chlorosis, may play a dominant role in iron transport into vessels (Kramer et al., 1980; Marschner et al., 1986; Römheld and Marschner, 1981). Transfer cells involved in iron translocation to shoots are located in i) stem nodes, where they favour the exchange of nutrients between adjacent but unconnected vascular bundles; ii) minor leaf veins, where they divert iron from xylem sap into the symplasm of adjacent cells; and iii) connecting zones of xylem and phloem, where they are involved in 'cross-traffic' of ions between the two tissues (Landsberg, 1984).

2.3.2. *Pigments and photosynthesis*

The most evident effect of iron deficiency is the decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls (Chl) and leading to the yellow colour characteristic of chlorotic leaves (Abadía and Abadía, 1993; Bassi et al., 1998; Miller et al., 1984; Morales et al., 1990, 1998b; Morales et al., 1994; Terry and Abadía, 1986).

Chlorophylls *a* and *b* are differentially affected by iron chlorosis. For instance, grapevine plants grafted on resistant rootstocks presented a smaller ratio Chl *a*:Chl *b* than those grafted on susceptible genotypes (Bavaresco et al., 1992). In Fe-deficient pear Morales et al. (1994) observed decreases in neoxanthin, β-carotene and Chl *a*, while lutein and carotenoids within the xanthophyll cycle were less affected. The pigments of the violaxanthin cycle (violaxanthin, antheraxanthin and zeaxanthin) seem to remain completely functional in Fe-deficient leaves since their epoxidation and de-epoxidation still occurs in response to light (Morales et al., 1990; Morales et al., 1994).

Due to the relative enrichment of carotenoids, the absorptance of pear and peach leaves decreased and the integrated reflectance and transmittance increased with iron deficiency (Abadía et al., 1999; Morales et al., 1991). In pear, leaf absorptance may decrease from control values of 80% to less than 45% in chlorotic leaves (Abadía et al., 1999; Morales et al., 2000b).

In Fe-deficient leaves, the number of granal and stromal lamellae per chloroplast decrease. This is associated with a decrease in all the components of the membrane, including electron transporters in the photosynthetic electron chain and light harvesting pigments – chlorophylls and carotenoids (Abadía and Abadía, 1993; Monge et al., 1993; Morales et al., 1994; Nedunchezhian et al., 1997; Pushnik and Miller, 1989; Quílez et al., 1992; Spiller and Terry, 1980; Terry, 1980; Terry and Abadía, 1986).

Iron chlorosis impairs the ultrastructure of chloroplasts (number of grana and stroma lamellar structures) but has little effect on other iron containing organelles such as peroxisomes and mitochondria (Hellín et al., 1995). Leaves of grape grown under iron deficiency showed fragmentation of the thylakoids and partial reduction of grana (Guller and Krucká, 1993).

Abadía et al. (1988) found changes in the lipid composition of pea leaves as a response to iron deficiency. The ratio of mono-galactosyldiglycerol to di-galactosyldiglycerol in thylakoids decreased in Fe-deficient plants (Monge et al., 1993). As a result, the thylakoids were more rigid in chlorotic than in green plants (Abadía et al., 1989b; Abadía, 1992). There is also a sharp decrease in thylakoidal iron content in plants affected by iron deficiency (Terry and Low, 1982).

The efficiency of photosystem II is only slightly affected by iron deficiency in leaves of orange trees, sugar beet and pear (Morales et al., 1991, 1998b; Pestana, 2000; Pestana et al., 2001c). The decrease in the ratio F_v/F_m (where F_v is the variable fluorescence, given by $F_v = F_m - F_o$, F_m is the maximum fluorescence, and F_o is the basal fluorescence) was associated with an increase in F_o, which could result from increases in the dark reduction of the plastoquinone pool (Belkhodja et al., 1994; Belkhodja et al., 1998a; Belkhodja et al., 1998b; Pestana et al., 2001c). Abadía

et al. (1999) concluded that, with the exception of severely chlorotic leaves, the remaining photosynthetic apparatus in leaves of Fe-deficient fruit trees does not present any photo-inhibitory damage, even at high densities of photosynthetic photon flux and with mild water stress, which represent the typical condition of crops growing in the Mediterranean area. The excess of light absorbed by Fe-deficient pear leaves was thermally dissipated within the antenna of photosystem II (Morales et al., 1998a; Morales et al., 2000a), mediated by the relative increase in xantho-phyll pigments (Abadía et al., 1999). These authors also referred the increased concentration of enzymes and other plant antioxidant defences able to scavenge 'acti-vated' oxygen. Chlorotic pear leaves showed down-regulation processes but not sustained photo-inhibition (Morales et al., 2000a, b). For more details see Abadía et al. (1999).

2.3.3. *Other enzymatic activities*

Several authors have reported the effect of iron deficiency on enzymatic activi-ties. For example, chlorophyllase – the enzyme responsible for *in vivo* degradation of chlorophyll – seems to have a greater substrate affinity in chlorotic than in green leaves of lemon (Fernandez-Lopez et al., 1992).

Lime-induced iron chlorosis in lemon led to decreases in the activity of perox-idase, catalase and the Fe-containing superoxide dismutase (Hellín et al., 1995; Hellín et al., 1983). However, there was a simultaneous increase in the activity of the superoxide dismutase that contained copper and zinc. This suggests an induction mechanism mediated by active oxygen species, as described by Abadía (1998). The enzymatic activities of catalase and peroxidase were correlated with the iron content in leaves (Ruiz et al., 2000).

The decline in the activity of ribonucleotide reductase, another Fe-containing enzyme (Schmidt and Schuck, 1996), hampers DNA synthesis and meristematic growth (Bañuls et al., 1993; Kosegarten et al., 1998a; Mengel, 1995). Iron deficiency also affects the level of active ribulose-1,5-biphosphate carboxylase/oxygenase (Winder and Nishio, 1995).

2.3.4. *Mineral composition*

The iron concentration in leaves required for optimal growth varies between species (Bavaresco, 1997; Marschner, 1995; Montañés et al., 1990b; Spiegel-Roy and Goldschmidt, 1996; Tagliavini et al., 1993): from 50 to 150 mg Fe kg^{-1} dry weight in peach, orange and apple, from 25 to 200 mg Fe kg^{-1} dry weight in cherry and plum, from 30 to 100 mg Fe kg^{-1} dry weight in blueberry, from 30 to150 mg Fe kg^{-1} dry weight in pear, and from 15 to 100 mg Fe kg^{-1} dry weight in petioles of grape. Iron contents less than these lead to iron chlorosis, and can be associated with other nutrient deficiencies.

The effects of lime-induced chlorosis on leaf mineral composition were studied in several fruit trees, such as apple (Ji et al., 1985; Tagliavini et al., 1992), peach (Abadía et al., 1985; Belkhodja et al., 1998b; Köseoglu, 1995a; Köseoglu, 1995b; Sanz et al., 1991, 1992), and lemon (Procopiou and Wallace, 2000). Results can

appear to be contradictory since plants vary in their requirements for nutrients. Moreover, the methods used in the assessment of nutritional status are sometimes very specific. A brief summary of some of the results obtained is presented next.

In lemon trees grown on calcareous soil, the iron concentration in leaves was related to the concentrations of phosphorus, potassium and manganese in leaves (Fernandez-Lopez et al., 1993).

In a field experiment with different pear rootstocks, Tagliavini et al. (1993) concluded that not only the uptake of iron but also manganese can be impaired by lime in soils, and that elevated copper levels can also induce iron chlorosis.

Romera et al. (1991c) observed the accumulation of manganese in young leaves of tolerant peach rootstocks growing in a nutrient solution without iron, but not in susceptible rootstocks. In field-grown peach trees, iron chlorosis lead to a sharp increase in the concentration of potassium in leaves, and to slight increases in nitrogen, magnesium and manganese, while phosphorus, copper and zinc were relatively unaffected by the chlorosis (Abadía et al., 1985; Belkhodja et al., 1998b; Köseoglu, 1995a; Köseoglu, 1995b). In nutrient solution, the peach rootstock 'Montclar' had only small concentrations of nitrogen, phosphorus, calcium and iron in the new branches grown in the presence of bicarbonate (Shi and Byrne, 1995; Shi et al., 1993a, b).

The different tolerance of several grafted grapevines became evident when iron uptake was expressed on a fresh weight basis. Total chlorophyll concentration was positively related to iron, calcium and magnesium, and negatively related to potassium contents of leaves (Bavaresco et al., 1992). According to Bavaresco (1997) the mineral composition of leaf blades and petioles of chlorotic and green leaves of grapevines were not significant different, but chlorosis seemed to affect the remobilisation of nitrogen, phosphorus, calcium and magnesium to the fruits.

3. DIAGNOSIS OF IRON CHLOROSIS IN FRUIT TREES

The evaluation of nutrient concentrations in plants is important in modern agriculture, not only to prevent potential deficiencies, but also as a powerful management tool to monitor the nutritional status of healthy crops. Based on a correct diagnosis it is possible to select the right type and amount of fertilizer and thus recommend a rational fertilizer programme, taking into account the risks of negative environmental impacts that can result from excessive applications of some nutrients.

Excesses or deficiencies of nutrients are a special concern in fruit trees, since in these crops nutritional imbalances can affect the yield for more than a single season. The agronomic consequences of iron chlorosis in fruit trees versus annual field crops were compared by Tagliavini et al. (2000). The differences in life cycle, plant size and characteristics of the root systems of trees, compared with annual plants, makes them more susceptible to iron chlorosis. In trees, iron deficiency affects the nutritional balance in the following year since new growth depends on iron stored in the plant. After absorption by roots and to reach the canopy, iron has to be transported a longer distance in the xylem of trees compared with annual crops. Impaired iron translocation may also result from a certain degree of scion-root-

stock incompatibility. The roots of fruit trees as they grow explore deeper soil layers that can have high levels of calcium carbonate and greater water content, factors that favour iron chlorosis. Also the root length density of trees is usually much less than in annual crops (Goss, 1991).

Since there are several types of iron chlorosis it is important to properly identify the cause in a particular situation. Therefore, both soil and plant analysis might be needed to investigate the origin of the problem.

3.1. Soil analysis

Soil analysis is routinely used as the basis for fertilisation recommendations of annual crops. However, soil tests have limited value when applied to trees because the root system is deep and unevenly distributed, making it difficult to obtain a representative soil sample.

Two major approaches can be taken to diagnose lime-induced iron chlorosis based on soil analysis (for a review see Hartwig and Loeppert, 1993), i) to analyse for available iron using extractants capable of chelating the metal, and ii) to determine the lime content of the soil. The active lime (Drouineau, 1942), i.e. the fine and reactive fraction of lime, can be used as an indicator of the risk of iron chlorosis, especially when the amount of extractable iron is also known. Rootstocks are ranked according to their tolerance to active lime, but very often susceptible-rootstocks have other characteristics that make them more eligible for commercial operations, such as tolerance to disease.

3.2. Plant analysis

Iron chlorosis can be identified by visual symptoms, a fast and economic method. Several authors proposed the use of visual scores, from 0 (without symptoms) to 5 (trees with dead branches and white young leaves) (McKenzie et al., 1984; Romera et al., 1991b; Sanz and Montañés, 1997). The degree of chlorosis can now be rapidly quantified by the measurement of chlorophyll content using a SPAD apparatus. However, by the time symptoms become apparent it is often too late to prevent the negative effects of the disorder on yield and fruit quality.

Tissue analysis offers a number of advances as well as some challenges.

3.2.1. *Leaf analysis*

Chemical plant analysis, in particular leaf analysis, is still the most common method used for diagnostic purposes in trees, and is based on the relationship between growth rate of plants and nutrient content (Moreno et al., 1998; Sanz and Montañés, 1995a, b). Leaf analysis integrates all the factors that might influence nutrient availability in the soil and plant uptake, and pinpoints the nutritional balance of the plant at the time of sampling. However, the use of leaf analysis presents limitations when applied to lime-induced chlorosis, since in many field-grown plants there is no correlation between leaf iron concentration and the degree of chlorosis expressed as chlorophyll content (Abadía, 1992; Hamzé and Nimah, 1982; Mengel et al., 1994;

Pestana et al., 2001b). Moreover, iron concentration in chlorotic leaves, expressed on a dry weight basis, is frequently even greater than in green leaves (Abadía, 1992; Aktas and Van Egmond, 1979; Bavaresco et al., 1993a; Bavaresco et al., 1999; Deckock et al., 1979; Fernandez-Lopez et al., 1993; Mengel, 1995; Morales et al., 1998c; Rashid et al., 1990; Terry and Low, 1982). This was called the 'chlorosis paradox' by Römheld (2000) and results from the inactivation of iron in leaves or from an inhibition of leaf growth due to iron chlorosis (Morales et al., 1998c). Morales et al. (2000c) observed a greater iron concentration in the petioles and veins of chlorotic leaves of peach trees than in the lamina, where active iron is located. In apple leaves under iron deficiency, Vedina and Toma (2000) observed a decrease in organic iron content, indicating low mobility of iron compounds.

Bavaresco et al. (1999) proposed the expression of iron concentration per leaf (µg Fe leaf^{-1}) rather than on a dry matter basis, as it allowed the separation of dark green from chlorotic leaves.

Another limitation of leaf analysis is the fact that the sampling date recommended for fruit trees is late in the growing season, generally very close to harvest. At this point it is no longer possible to correct nutritional disorders in time to avoid decreases in yield (Sanz and Montañés, 1995b). In fact, according to Igartua et al. (2000), at the recommended date for foliar analysis of peach, 120 days after full bloom, most of the varieties grown in the Mediterranean area are already harvested or are very close to harvest. This also happens with pear (Sanz and Montañés, 1995b). It is therefore important to develop a useful method to diagnose iron deficiency in fruit trees before yield is affected.

The standard method used to interpret the results of leaf analysis is to compare nutrient concentrations to reference values for a particular crop and sampling method. At most, this procedure can identify a single deficiency at a time, but does not evaluate the nutrient balance. Due to the complexity of the nutritional imbalances resulting from iron chlorosis, several authors have proposed the use of indexes to interpret plant analysis data. These include i) nutrient ratios, ii) Diagnosis and Recommendation Integrated System (DRIS), and iii) Deviation from Optimum Percentage (DOP) (Beverly et al., 1984; Guzmán and Romero, 1988; Guzmán et al., 1991; Köseoglu, 1995b; López-Cantarero et al., 1992; Montañés and Heras, 1991; Valenzuela et al., 1992).

The use of nutrient ratios to interpret foliar analysis was proposed for apple (Tagliavini et al., 1992), peach (Abadía et al., 1985; Alcántara and Romera, 1990; Köseoglu, 1995a), quince (Tagliavini et al., 1995b), pear (Tagliavini et al., 1993), citrus (Fernández, 1995; Hellín et al., 1984; Wallace, 1990), grape (Bavaresco, 1997), and berries (Bavaresco, 1997). The nutritional relationships identified were the ratios P:Fe (Köseoglu, 1995a; Mengel et al., 1984; Wei et al., 1995), K:Ca (Abadía et al., 1989b; Abadía et al., 1985; Garcia et al., 1999; Mengel et al., 1984; Montañés et al., 1990a), Fe:Mn (Lucena et al., 1990; Monge et al., 1993) and Zn:Fe (Nenova and Stoyanov, 1999). These ratios express nutritional imbalances that appear when iron immobilization in the plant takes place. However, no absolute values could be established for any of the ratios to enable the diagnosis of iron chlorosis under field conditions (Chaney, 1984).

The analysis of an 'active' pool of iron in leaves (usually identified with Fe

(II)), using extractants such as acetic, nitric and hydrochloric acids, 2,2′ bipyridyl and o-phenanthroline, is frequently mentioned (Abadía et al., 1989b; Bavaresco et al., 1993a; Mohamed et al., 1998; Rashid et al., 1990). However, according to Abadía (1992) this method does not solve the problem adequately because these extractants may also remove some Fe (III) from leaves, such as the iron in phytoferritin. In peach, it was estimated that 2,2 bipyridyl and o-phenanthroline extracted 2 of 4 nmol Fe cm^{-2} from severely chlorotic leaves, and 4 of 7 nmol Fe cm^{-2} from controls (Abadía et al., 1985; Zohlen, 2000).

In the DRIS method the nutritional status of a high yielding population is described and used to identify variations in the nutrient balances of other plant samples (Beverly et al., 1984). The mathematical equations needed for the calculation of limiting nutrients are described in detail by Beaufils (1973). This method has already been applied successfully to citrus (Beverly et al., 1984; Malavolta et al., 1993; Moreno et al., 1996) and peach (Sanz, 1999). However, it is somewhat complex and expensive because of the very large data bases required to obtain reliable results. The compositional nutrient approach, a modified DRIS-system, was tested in grape and can be used to optimise fertilizer inputs (Schaller et al., 2001).

As an alternative to DRIS, Montañés and Heras (1991) introduced the DOP index, which provides information from quantitative and qualitative perspectives. As developed by Montañés et al. (1993) the DOP index is calculated for each nutrient by a simple equation:

$$\left(\frac{100\ C}{Cref}\right) - 100$$

where C is the nutrient concentration in the sample under study, and *Cref* is the optimum nutrient concentration, both expressed on a dry matter basis. Negative and positive DOP indices, respectively, will result when a deficiency or excess of the nutrient occurs. With this method it is possible to make a list of the nutrients that are limiting yield (Sanz, 1999).

The Fe-index, derived from the DOP approach, is calculated from the equation:

$$\frac{(10C + K)50}{Fe}$$

where P and K are the concentrations of these nutrients, expressed as % in the dry matter, and *Fe* is the iron concentration expressed as µg Fe g^{-1} dry weight. With this index either total or active iron can be used. The Fe-index has been successfully applied in horticultural crops, where high values of the index were found in chlorotic leaves (Guzmán and Romero, 1988; Guzmán et al., 1991; Valenzuela et al., 1995). The index was also applied to fig, using both total and soluble iron, and to plum (Moreno et al., 1998; Romero, 1992).

Using mineral analysis the level of a nutrient is determined but it is seldom possible to distinguish metabolic (active) forms from non-active (Bar-Akiva, 1964). To overcome this difficulty, some researchers have measured key enzymatic activ-

ities to diagnose iron chlorosis in fruit trees. Garcia and Galindo (1991) proposed the use of chlorophyllase activity as a biochemical indicator of manganese and iron deficiencies in citrus. In leaves of lemon, iron deficiency decreases peroxidase, catalase and some superoxide dismutase activities (Hellín et al., 1995), enzymes that are part of the intrinsic enzymatic defensive system required for the detoxification of superoxide radicals. The enzymatic methods may become a valuable tool to establish the nutritional status of plants, but further work is needed to support these early findings (Lavon and Goldschmidt, 1999).

3.2.2. *Floral analysis*

As a novel approach for the prognosis of iron deficiency in pear trees Sanz et al. (1993) proposed methods based on the mineral composition of flowers. These authors stated that floral analysis could be used to determine the nutritional status of crops at an early stage, since the mineral composition of flowers at full bloom is often related to the nutrient content (of the same nutrients) in leaves taken 120 days later. Flower analysis has now been developed for a number of fruit trees: pear (Sanz et al., 1993; Sanz and Montañés, 1995b; Sanz et al., 1994), peach (Belkhodja et al., 1998b; Igartua et al., 2000; Sanz et al., 1997a; Sanz and Montañés, 1995b), nectarine (Toselli et al., 2000), apple (Morales et al., 1998c; Sanz et al., 1998), walnut (Drossopoulos et al., 1996), olive (Bouranis et al., 1999), pistachio (Vemmos, 1999), almond (Bouranis et al., 2001), and citrus (Pestana et al., 2001b).

The main advantage of analysing flowers over leaves is that the evaluation can take place earlier in the season. The recommended date for foliar analysis of fruit trees is mid-summer, because this is the period of greatest stability for leaf nutrient concentrations (Spiegel-Roy and Goldschmidt, 1996). In contrast, when based on flower analysis the nutritional diagnosis of fruit trees can be advanced to April (Abadía et al., 2000; Igartua et al., 2000; Sanz et al., 1992). In deciduous trees, flowers will be sampled even before leaf emergence and their mineral content expresses the nutritional status of the tree (Abadía et al., 2000). In both deciduous and evergreen fruit trees, using floral analysis it is possible to detect and correct any deficiencies before fruit set, thus giving sufficient time for nutrient amendments to improve yield and fruit quality (Belkhodja et al., 1998b; Igartua et al., 2000; Sanz et al., 1997b; Sanz et al., 1998).

To diagnose iron chlorosis based on the iron content of flowers, results must allow the prediction of leaf chlorophyll later in the season. This has indeed been demonstrated for several species, albeit that correlation coefficients were in same cases as small as 0.50 (Table 1). For example, after long-term experiments Sanz et al. (1997b) stated that the probability of iron chlorosis developing in peach trees is large when the concentration of iron in flowers is less than 160 mg kg^{-1} dry weight.

However, there have been several cases where no correlation was found between iron concentration in flowers and leaf chlorophyll later in the season. Abadía et al. (2000) stated that most, if not all, of the iron present in flowers of deciduous trees in full bloom was already present in the tree when it was dormant. The application of fertilisers after flowering may facilitate iron uptake and increase iron supply to leaves, even if iron is not applied (for example when ammonium nitrogen is used).

Table 1. Iron concentrations in flowers of several fruit trees (mean, minimum and maximum), and correlation coefficients (*r*) of the regressions between iron in flowers (mg kg^{-1} DW) and leaf chlorophyll (Chl) 120 days after full bloom.

Fruit tree	Cultivars	Flower Fe		*r*	Authors
				Flower Fe vs.	
		mean	min-max	leaf Chl	
Nectarine	Spring Red	65	34–82	0.50*	(Toselli et al., 2000)
Apple	Golden Delicious	388	250–544	0.88*	(Sanz et al., 1998)
Peach	Babygold 7	293	145–573	0.74**	(Sanz et al., 1997b)
Orange	Valencia late	41	16–69	–0.51*	(Pestana, 2000)

Significance level: * $p < 0.01$; ** $p < 0.001$.

This would likely result in a greater chlorophyll content than a simple prediction from iron in the flowers. On the other hand, the heterogeneity frequently observed in the degree of chlorosis, even in a single tree, can prevent the establishment of a relationship between iron in flowers and level of chlorophyll in leaves (Sanz et al., 1993).

The interpretation of floral analysis is thus as complex as when leaf analysis is carried out, and requires similar tools to obtain a correct diagnosis. Rather than the use of a singular concentration, nutrient balances are now being investigated in the search for a good indicator of iron chlorosis. The pattern of iron accumulation in fruit trees seems to depend on their life cycle. In deciduous species (nectarine, peach, pear and apple trees), the mean concentration of iron was greater in flowers than in leaves, contrary to what was observed in orange (Pestana, 2000; Pestana et al., 2001b; Sanz et al., 1993). There was also a greater degree of variation in the range of values obtained in flowers of deciduous trees, contrasting with those obtained in orange trees. Significantly, the correlation coefficient, r, for the relationship between iron in the flowers and the chlorophyll in the leaves was positive for the deciduous trees but negative for orange (cv. 'Valencia late') (Table 1). While in deciduous trees flowering occurs before vegetative growth, full bloom in orange generally occurs in April in Portugal, concurrent with new vegetative growth. Young leaves are thus likely to act as strong sinks for iron in citrus, and compete with translocation towards flowers. Probably as a result of these differences, the concentrations or ratios of nutrients that can be used as indexes vary between species.

Examples of nutrients and balances related to iron chlorosis are the increase in potassium content and in the K:Ca ratio resulting from lime-induced chlorosis in flowers of peach (Belkhodja et al., 1998b). Moreover, while the iron concentration in flowers of peach fluctuates from year to year, a major problem when using this element for the prognosis of the chlorosis later in the year, the concentrations of potassium and zinc and the K:Zn ratio in flowers had consistent values from year to year, making them more likely candidates as indicators of iron chlorosis (Abadía et al., 2000; Igartua et al., 2000). The physiological basis for the changes in potassium are possibly associated with increases in the activity of plasmalemma ATPases involved in proton extrusion by roots and accumulation of organic acids in Fe-deficient plants (Igartua et al., 2000). Zinc may share with iron the acquisi-

tion and translocation mechanisms in the plant (Grusak et al., 1999). In agreement with this, Igartua et al. (2000) proposed the use of the ratio K:Zn to predict iron chlorosis later in the year. A K:Zn ratio over 450 in flowers at full bloom is likely to be associated with the development of iron chlorosis in peach (leaf chlorophyll concentrations below 200 µmol m^{-2}) 120 days later, while chlorosis is unlikely to develop with a ratio below 375.

4. CORRECTION OF IRON CHLOROSIS IN FRUIT TREES

The correction of iron chlorosis in plants grown on calcareous soils is an old problem with no easy solution (Chandra, 1966; Démétriadrès et al., 1964). Until rootstocks tolerant to iron chlorosis and with other favourable agronomical characteristics become available, the prevention or correction of iron chlorosis is of paramount importance to fruit growers. Obviously, the need to correct iron chlorosis is related to its effects on yield, fruit size and quality, and consequently to decreases in the growers' profits.

In a recent review Tagliavini et al. (2000) summarized the economical impact of iron chlorosis in kiwi, peach and pear orchards established on calcareous soils in Italy, Spain and Greece and concluded that yield losses were directly related to the intensity of iron chlorosis. A significant proportion of peaches and kiwifruit were unsuitable for the market. However, Sanz et al. (1997b) found that iron chlorosis only affected peach quality when visual symptoms were obvious, corresponding to a severe deficiency.

In another study, the reduction of yield due to iron deficiency in kiwi was estimated as about 50%, mainly as a consequence of the reduction in the number of fruits per plant, rather than smaller fruit size (Loupassaki et al., 1997).

El-Kassa (1984) reported the negative effect of iron chlorosis on gross yield and fruit quality of lime, resulting in smaller fruit that were more acidic and contained less ascorbic acid. The correction of iron chlorosis with sprays containing iron resulted in larger oranges, representing a gain of more than 35% in the gross income of the farmer (Pestana et al., 2001a). Furthermore, iron chlorosis can lead to a delay in fruit ripening in orange and peach (Pestana, 2000; Pestana et al., 2002; Pestana et al., 2001a; Sanz et al., 1997b).

The treatments already tested for the correction of iron chlorosis can be applied directly to soils or to the plants as foliar sprays.

4.1. Treatments applied to soils

The correction of iron chlorosis in trees grown on calcareous soils is normally achieved by the application of Fe (III)-chelates such as iron ethylenediaminedi-*o*-hydroxyphenylacetate (Fe-EDDHA) to the soil (Legaz et al., 1992; Papastylianou, 1993). This practice is very expensive and has to be repeated every year because iron is rapidly immobilized in the soil or leached out of the root zone. Tagliavini et al. (2000) estimated a cost of 250 Euros per hectare, accounting for up to 60% of total fertilizer costs. Moreover, chelating agents remain in the soil after Fe^{2+} uptake

by plants, and become available to react with other metals, such as manganese, copper and nickel, thus increasing their bioavailability (Wallace et al., 1992). The efficacy of treatments with Fe-EDDHA is related to the great stability of this chelate, even when the soil pH is above 9, preventing the precipitation of iron (Andréu et al., 1991; Hernández-Apaolaza et al., 1995; Lucena et al., 1992a, b; Wallace, 1991). In contrast, the stability of iron ethylenediamine-tetraacetate (Fe-EDTA) decreases above pH 6.5, resulting in the exchange of iron by others cations, such as Ca^{2+}, Zn^{2+} and Cu^{2+}, and in the precipitation of iron. Therefore, the application of Fe-EDTA to alkaline soils is not effective (Alva, 1992b). The application of Fe-chelates to soils is time consuming since they are placed around each individual tree, normally in the spring between the beginning of flowering and full bloom (Rombolà et al., 1999). Iron chelates can also be applied during the autumn-winter period, delaying the appearance of iron chlorosis in the spring, but the chelates are easily leached out of the rooting zone due to heavy rain during this period (Papastylianou, 1993; Tagliavini et al., 2000).

Large amounts of iron have to be applied each time, since the use efficiency of the nutrient is always very small. For example, to correct lime-induced chlorosis in citrus rates of 10 to 25 g per tree are needed (Legaz et al., 1992).

Several studies have attempted to overcome iron chlorosis with soil treatments that do not involve synthetic chelates. The increase of iron availability in the rhizosphere can be achieved with the addition of other iron compounds or through changes in rhizosphere conditions.

Iglesias et al. (2000) effectively prevented iron chlorosis in pear trees grown in a calcareous soil, by injecting a synthetic Fe (II) phosphate $(Fe(PO_4)_2.8H_2O)$ in the soil.

The addition of Fe (II) sulphate alone to calcareous soils is not effective since iron precipitates and becomes unavailable to plants (Loeppert, 1986; Ruiz et al., 1984), but its effectiveness can be enhanced when added with organic matter. Organic matter can prevent or correct lime-induced chlorosis due to complexation and solubilisation of iron (Horesh et al., 1986; Wallace, 1991), though the efficacy of the treatment depends on the organic matter composition, capacity to complex iron, and stability of the Fe-chelates formed (Hagstrom, 1984).

In a pear orchard established on a calcareous soil Tagliavini et al. (2000) obtained the recovery from iron chlorosis with the application of blood meal or of compost enriched with $FeSO_4$. These authors also referred to similar results with manure applied to a peach orchard and attributed them to the ability of humic and fulvic substances to chelate iron, and to the fact that roots could grow into the organic matrix and absorb iron from microsites where lime was absent.

The use of industrial by-products and wastes has also been tested in herbaceous species with varying degrees of success (Hagstrom, 1984). According to Alva (1992a) iron humate, a by-product of the drinking water decolourisation process was an effective source of iron for citrus trees planted on alkaline soils. In a follow-up study Alva and Obreza (1998) reported the increase in growth, leaf iron concentration, and fruit yield following the application of iron humate. Incorporation of sewage sludge and a hydrogel significantly improved the growth of apple seedlings (Awad et al., 1995a).

The prevention of lime-induced chlorosis by acidification of the entire root zone is unrealistic (Tagliavini et al., 1995a; Wallace, 1991). Less than complete neutralization would have little or no effect on the chlorosis; the amount of sulphur or sulphuric acid needed to achieve complete neutralization would be enormous, and the appropriate application rate would need to be varied according to the lime content within the soil profile. Broadcast application of small amounts of strong acids to calcareous soils does not significantly decrease pH, and may have negative effects, namely phytotoxity and increased soil salinity (Khorsandi, 1994). In contrast, local acidification of small volumes of soil is possible and can significantly improve the nutritional status of fruit trees. Horesh et al. (1986, 1991) corrected lime-induced chlorosis in citrus, with the application of a peat-plug with iron sulphate to small volumes of soil close to the trees. The recovery from iron chlorosis of citrus grown on a calcareous soil in Florida was obtained with the application of Fe-EDTA (57g of Fe per tree) and concentrated sulphuric acid to six holes dug around each tree (Obreza et al., 1993). Application of elemental sulphur, banded on both sides of tree rows, allowed for excellent chlorosis control in peach trees and simultaneously improved the availability of phosphorus, manganese and zinc to plants (Wallace, 1991).

In calcareous soils nitrogen nutrition is predominantly based on nitrate even when ammonium is applied due to rapid nitrification, but rhizosphere acidification can still be achieved when nitrification inhibitors are used with urea or ammonium (Tagliavini et al., 1995a). Recently, a promising technique based on the Controlled Uptake Long Term Ammonium Nutrition (CULTAN) cropping system established by (Sommer, 1992) has been adapted to prevent and control lime-induced iron chlorosis (Jaegger et al., 2000). In this system, small amounts of soil in the rooting zone are replaced with a mixture of compost and sandy soil with a pH of 2.0 to 3.0 (due to addition of sulphuric acid). In the same location ammonium sulphate with a nitrification inhibitor and iron sulphate are applied.

4.2. Treatments applied to trees

Foliar sprays can be a cheaper and environmental-friendly alternative to soil treatments to control iron chlorosis. Applying iron compounds or acid solutions to shoots bypasses the inhibitory effects of soil bicarbonate on iron uptake and translocation (Mengel, 1995; Wallace, 1995). Release of iron immobilized in the plant can also be achieved (Tagliavini et al., 1995a; Tagliavini et al., 1995c). The success of treatments with iron compounds depends on their capacity to penetrate the cuticle, travel through the apoplastic free space and cross the plasmalemma of leaf cells to reach the cytoplasm (Rombolà et al., 2000).

The foliar application of Fe (II) sulphate increased leaf chlorophyll content in kiwi (Rombolà et al., 2000) and citrus (Hamzé et al., 1985; Horesh and Levy, 1981; Miller et al., 1994; Pestana et al., 2002; Pestana et al., 2001a; Pestana et al., 1999). Though this treatment can improve fruit size and quality, as observed in orange (Pestana et al., 2002; Pestana et al., 2001a; Pestana et al., 1999), the positive effects obtained on leaf chlorophyll content did not always translate into

increased yield, because the translocation of the applied iron into developing new leaves or fruits can be small (Legaz et al., 1992).

Several authors tested foliar applications of iron chelates to plants such as orange (El-Kassa, 1984; Legaz et al., 1992; Pestana et al., 2002; Pestana et al., 2001a), tangerine (Pestana et al., 1999); grape (Cuesta et al., 1993), and kiwi (Rombolà et al., 2000; Rombolà et al., 1998b; Tagliavini et al., 2000). The foliar application of chelates can be less efficient than soil application, due to limited uptake by aerial parts (Legaz et al., 1992), but the results obtained by Rombolà et al. (2000) suggest that leaves of field-grown kiwi were able to reduce the Fe (III) from diethylenetrianinepentaacetic acid (DPTA) and take it up into mesophyll cells. This is also true for citrus (orange and tangerine) since the recovery from iron chlorosis symptoms was obtained after frequent foliar sprays with Fe (III) from Fe-EDDHA (Pestana, 2000; Pestana et al., 2002; Pestana et al., 2001a; Pestana et al., 1999).

Other treatments that can be applied directly to trees are products that promote the activity of the Fe-chelate reductase present in the plasmalemma of leaf meso-phyll cells. Examples are dilute solutions of mineral or organic acids, hormones, alcohols and urea. Acid treatments release the iron immobilized within the plant by changing apoplastic pH (Tagliavini et al., 1995a; Tagliavini et al., 1995c). Sprays with sulphuric, citric and ascorbic acids on their own have been assayed in kiwi, pear and orange, but resulted in an incomplete recovery of the symptoms of iron chlorosis (García et al., 1998; Pestana et al., 2002; Pestana et al., 2001a; Pestana et al., 1999; Rombolà et al., 1998b; Tagliavini et al., 1995c). Supplementation of acid solutions by iron sulphate increased the efficacy of the treatment since the iron concentration in leaves can be enhanced by the mobilization of the iron already present and by applied iron (García et al., 1998; Rombolà et al., 1999; Varennes et al., 1997).

Application of substances that stimulate proton pumps located in the plasma-lemma should also alleviate iron chlorosis, based on the concept outlined by Mengel (1995) that the leaf apoplast affects the activity of the Fe (III)-chelate reductase. Mengel et al. (1984) treated chlorotic maize leaves with sprays containing fusic-occin and indole-3-acetic acid (IAA). Tagliavini et al. (2000) applied IAA (50 μmol L^{-1}) to kiwi grown in calcareous soils, which resulted in enhanced chloro-phyll content.

Sahu et al. (1987) tested the effects of sprays with different chemicals on the chlorophyll concentration and yield of peas grown in pots containing calcareous soil. The best result corresponded to the treatment with sulphuric acid followed by 'Mixtafol', which is a mixture of long-chain aliphatic alcohols.

The results obtained by the application of iron complexed by polyflavonoids were inconsistent in peach and plum (Spiegel-Roy, 1968), but in lemon promising results were reported (Fernandez-Lopez et al., 1993; Hellín et al., 1984).

Rombolà et al. (2001) reported the application of plant extracts on pear grown in pots filled with a calcareous soil. The extracts were obtained by maceration in water of several species such as *Amaranthus retroflexus, Beta vulgaris, Chenopodium album* and *Urtica dioica*. The best regreening was obtained after application of an

extract of *Amaranthus retroflexus* mixed with $FeSO_4$, resulting in a chlorophyll concentration similar to that obtained with Fe-chelate treatments to soils or leaves.

Table 2 summarizes the re-greening effects obtained by the foliar application of several compounds to various fruit trees.

The different results obtained with foliar application of the same product to different species may derive from differences in leaf permeability, dependent on

Table 2. The re-greening effects obtained by the foliar application of several compounds at different concentrations to some fruit trees.

Species	Compounds	Concentration	Re-greening	Authors
Kiwi	Fe (III) DTPA	72 mg Fe L^{-1}	Total	(Rombolà et al.,
	Citric acid (CA)	2 g L^{-1}	Partial	2000; Rombolà
	Fe (II) sulphate (IS)	207 mg Fe L^{-1}	Total	et al., 1998a;
	CA + IS	2 g L^{-1} + 207 mg Fe L^{-1}	Partial	Tagliavini et al.,
	Sulphuric acid (SA)	100 mg L^{-1}	Partial	2000)
	SA + IS	100 mg L^{-1} + 207 mg Fe L^{-1}	Partial	
	Indole-3-acetic acid	50 µmol L^{-1}	Partial	
	Fe (III) malate	1 mM; 3 mM	Partial	
	Fe (III) citrate	1 mM; 3 mM	Partial	
	Fe (III) DTPA	2 mM Fe	Total	
	IS + bioproteins (a)	325 mg ml^{-1}	Total	
	Fe (III) EDTA	10 g L^{-1}	No	(Loupassaki et al.,
	(Fe + Mn) EDTA	10 g L^{-1} + 10 g L^{-1}	Partial	1997)
Peach	Polyflavonoid Fe	9.6% Fe DW	No	(Spiegel-Roy, 1968)
Pear	Citric acid	2 g L^{-1}	No	(Rombolà et al.,
	Fe (II) sulphate (IS)	207 mg Fe L^{-1}	Partial	2000; Rombolà
	CA + IS	2 g L^{-1} + 207 mg Fe L^{-1}	Partial	et al., 1998a;
	IS + bioproteins (a)	325 mg ml^{-1}	Total	Tagliavini et al., 2000)
	Ascorbic acid (AA)	2 g L^{-1}	Partial	(García et al., 1998)
	Citric acid	2 g L^{-1}	Partial	
	Sulphuric acid (SA)	0.55 g L^{-1}	Partial	
	Fe (III) DTPA	199 mg Fe L^{-1}	Total	
	Fe (II) sulphate (IS)	500 mg Fe L^{-1}	Total	
	AA + IS	2 g L^{-1} + 500 mg Fe L^{-1}	Partial	
	CA + IS	2 g L^{-1} + 500 mg Fe L^{-1}	Partial	
	SA + IS	0.55 g L^{-1} + 500 mg Fe L^{-1}	Partial	
Apple	Polyflavonoid Fe	9.6% Fe DW	Total	(Spiegel-Roy, 1968)
Orange	Fe (III) EDDHA	120 mg Fe L^{-1}	Total	(Pestana et al.,
	Fe (II) sulphate	500 mg Fe L^{-1}	Total	2002; Pestana et al.,
	Sulphuric acid	0.5 mM	Partial	2001a)
Tangerine	Fe (III) EDDHA	120 mg Fe L^{-1}	Total	(Pestana et al.,
	Fe (II) sulphate	500 mg Fe L^{-1}	Total	1999)
	Sulphuric acid	0.5 mM	Partial	

Total and partial regreening are relative effects by comparison with application of Fe (III)-chelates; (a) Iron complexed with aminoacids and polypeptides. DTPA – diethylenetrianinepentaacetic acid; EDTA – ethylenediaminetetraacetic acid; EDDHA – ethylenediamine-*o*-hydroxyphenylacetic acid.

cuticle composition and thickness, and response mechanisms to iron deficiency (Rombolà et al., 2000).

According to (Tagliavini et al., 2000) the activation of iron pools in chlorotic leaves rarely results in a full recovery from iron chlorosis because part of the iron is inactivated on the outside of mesophyll cells. Therefore, foliar treatments are only effective in situations with slight or moderate symptoms of iron chlorosis, and the effect is short-lived requiring repeated applications to maintain the regreening of leaves (Rombolà et al., 2000).

4.3. Other treatments

Data presented by several authors (Heras et al., 1976; Ruiz et al., 1984; Toselli et al., 1995; Wallace, 1991) shows that injection of ferrous sulphate into tree trunks can correct iron chlorosis, but this is an expensive procedure and the wounds that are caused in the tree represent an increased risk of bacterial or viral infections.

A nutrient solution containing macro and micronutrients dissolved in methanol was applied by Nonomura et al. (1995) directly on the bark of the larger stems of young citrus trees. Iron deficiency was corrected, probably due to the effect of methanol on nutrient uptake.

On calcareous soils with only small concentrations of active lime, the use of an integrated management system can be effective in dealing with iron chlorosis. Minimal tillage, especially during the rainy season, allows the establishment of grasses that improve soil infiltration and hydraulic conductivity, and release phytosiderophores to the rhizosphere (Toselli et al., 1995). These effects improve soil aeration and iron chelation increasing the bioavailability of the nutrient. Tillage seems to be necessary only when there is a strong competition for nutrients and water between grasses and fruit trees.

Toselli et al. (1995) identified *Lolium perenne* L., *Poa pratensis* L., *Festuca rubra* L., and *F. ovina* L. as an example of a mixed sward that can be sown around fruit trees. In a mature pear orchard Tagliavini et al. (2000) sowed a mixture of grasses (mainly *Poa* spp., *Lolium* spp. and *Festuca* spp.) along the tree rows and amended the soil with iron sulphate or iron chelate.

Another practice that can be implemented is the use of fertilizers with acidic reactions, like potassium sulphate (Mengel, 1995; Wallace, 1991).

Fertigation supplies nutrients to crops through irrigation water. The efficacy of iron application by fertigation depends on the bicarbonate level of the water, and on the form of iron. Lucena et al. (1991) stated that the simultaneous application of two types of chelates (Fe-EDTA and Fe-EDDHA) by fertigation allowed sustained high levels of iron in solution. Zekri and Koo (1992) reported the positive effects of Fe-chelate applied by fertigation to citrus. Rombolà et al. (2000) observed similar results after applying Fe-chelate to kiwi. Tagliavini et al. (1995a) reported that the use of ammonium sulphate in a liquid form (e.g. by fertigation) led to soil acidification and to enhanced micronutrient availability.

Several authors (Jurkevitch et al., 1992; Walter et al., 1994) claim that siderophores are an important source of iron for plants growing on calcareous soils. Root colonization by *Pseudomonas fluorescens* and *Glomus mosseae* led to

an increase in leaf iron in grape (cv. 'Chardonnay') grafted on a chlorosis-susceptible rootstock (Bavaresco et al., 1995b). Leaf chlorophyll concentration was directly correlated with the extent of root arbuscular micorrhyzal fungi infection of ungrafted rootstocks of grape (Bavaresco et al., 2000a; Bavaresco et al., 2000b). These results are promising but further research is needed to understand the role of symbionts on iron availability, specifically for fruit trees grafted on different rootstocks and grown on calcareous soils.

Ultimately, correction of iron chlorosis should not substitute for research to breed genotypes with better iron use efficiency. However, rootstocks must also perform well in other aspects, particularly in terms of resistance to pests and diseases. In fruit trees, information on the mechanisms of response to iron chlorosis is poor, but there is some evidence of resistance genes in some species that could be used in breeding programmes (Socias i Company et al., 1995).

4.4. Tolerance of rootstocks to iron chlorosis

Rootstocks exhibit different tolerances towards iron deficiency in calcareous soils. In general, non-trifoliate rootstocks of citrus are tolerant, while pure trifoliate (*Poncirus trifoliata* L. Raf.) rootstocks are very susceptible to lime-induced chlorosis (Byrne et al., 1995; Sudahono et al., 1994). For example, in a study carried out at two locations in southern Texas, Byrne et al. (1995) concluded that the most tolerant rootstocks were *Citrus obovoídea* Hort. × *Takahashi* (Kinkoji), *C. canaliculata* Tan., Texas sour orange (*C. aurantium* L.), Tosu sour orange (*C. neo-aurantium* Tan.), Cleopatara mandarin (*C. reticulata* Blanco), Schaub rough lemon, standard rough lemon, Vangasay lemon (*C. limon* L. Burm.), 1578-201 (*C. sinensis* L. Osbeck × *C. jambhiri* Lush.), Sunki mandarin × Swingle trifoliate (*C. reticulata* × *P. trifoliata*), and Shaddock × Rubidoux trifoliate (*C. grandis* Osbeck × *P. trifoliata*). The most susceptible rootstocks were Rangpur lime × Swingle trifoliate (*C. limonia* Osbeck × *P. trifoliata*), Cleoptara mandarin × Rubidoux trifoliate (*C. reticulate* × *P. trifoliata*), Sunki mandarin × Benecke trifoliate (*C. reticulate* × *P. trifoliata*), Benton citrange (*C. sinensis* L. Osbeck × *P. trifoliata*), and the three trifoliates (Flying Dragon, Pomeroy, and Argentine). However, tolerance to other factors such as the tristeza virus limits the choice of rootstocks that can be used.

Among grape rootstocks, hybrids from *Vitis berlandieri* × *Vitis rupestris* '140 Ru' and *V. berlandieri* × *V. riparia* 'SO4' are tolerant to lime-induced chlorosis, while hybrids from *V. riparia* × *V. rupestris* are susceptible (Bavaresco et al., 1995a; Bavaresco et al., 1994, 1995b).

According to Socias i Company et al. (1995) apple, grafted on apple roots, is tolerant to iron chlorosis, but for pear the situation is more complex since clonal quinces, seedling pears and clonal pears can all be used as rootstocks.

The most tolerant rootstocks that can be used with *Prunus* species are peach and almond hybrids, and the most susceptible is *Prunus persica* cv. 'Nemaguard' (Shi and Byrne, 1995). Tagliavini and Rombolà (2001) provide a more detailed review of differences in tolerance to iron chlorosis between rootstocks.

To reduce the time period needed to obtain improved plants Jolley and Brown (1994) proposed the use of screening methods based on physiological responses

of plants to iron chlorosis, which can be applied to young plants. Gogorcena et al. (2000) proposed the use of root Fe (III)-chelate reductase activity to screen peach rootstocks, as did Tagliavini et al. (1995b) for pear and quince rootstocks. Dell'Orto et al. (2000) evaluated the tolerance to iron chlorosis of new interspecific grape hybrids by their ability to acidify the medium and to reduce iron.

In vitro culture can also be used to screen genotypes for tolerance to iron chlorosis, as proposed for grape (Bavaresco et al., 1993b), quince (Muleo et al., 1995), citrus (Shijiang et al., 1995), and onion (Tisserat and Manthey, 1996). This methodology can be adopted on a small scale, and can be used to elucidate plant responses and to induce somaclonal variation in breeding programmes.

The isolation of the *FRO2* gene in Fe-deficient roots of *Arabidopsis* by Robinson et al. (1999) may hasten the creation of crops with improved iron acquisition and enhanced growth under Fe-deficient conditions. *FRO2* belongs to a family of flavocytochromes that transport electrons across membranes and seem to be related to iron tolerance. However, additional information on morphological, physiological and molecular mechanisms involved in the different genetic responses to iron chlorosis is still required. In fruit trees, screening methods must also consider that the behaviour of plants used as rootstocks may be different when ungrafted than when a scion has been grafted.

5. CONCLUSIONS AND OUTLOOK

Undoubtedly, there has been a major improvement in the understanding of lime-induced iron chlorosis over the last 15 years. Nevertheless, several aspects remain unclear, especially when related to fruit trees grown under field conditions.

The mobilization of soil iron and the role of microorganisms in iron acquisition, require more investigation. Iron fluxes in fruit trees grown on calcareous soils need to be studied as they may lead to reduced iron applications and new alternatives to control iron chlorosis. To overcome iron chlorosis, additional attention should be paid to the use of mixed crops and to application of organic residues to soil.

Due to the 'chlorosis paradox' flower analysis appears to offer major advantages such that it may substitute for leaf analysis in diagnosis of iron chlorosis, but more information is needed before it can be used to assess the nutritional status of all fruit trees. A greater understanding of the involvement of hormones on the adaptive mechanisms to iron chlorosis in tolerant species is needed, including the study of wild species well adapted to iron starvation.

More emphasis should also be put into the management of calcareous soils. The use of an integrated management system to correct iron chlorosis should consider economic, ecological and social aspects. Orchard management techniques are sustainable only if they represent an advantage for fruits growers, and the studies on iron chlorosis should include the effects on fruit quality and yield.

Genetically improved chlorosis-resistant rootstocks still offer the best solution to iron chlorosis, but this is a long-term approach. Screening techniques to identify tolerant genotypes need to be further developed. However, additional information

on morphological, physiological and molecular mechanisms involved in the different genetic responses to iron chlorosis is still required.

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Si IN HORTICULTURAL INDUSTRY

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1. INTRODUCTION

Si is one of the most widely distributed elements in the Earth's crust, and in turn soil is the most enriched with silica layer of the Earth's crust – 40 to 70% of SiO_2 contain in the clay soils and 90–98% in the sandy soils. Mainly, Si is present as quartz, alkali and aluminum silicates. They usually form the soil skeleton and are chemically or biochemically inert (Perelman, 1986; Reims, 1990; Sokolova, 1985). In the classification of element mobility, Si is defined both as an inert and as a mobile element (Perelman et al., 1989). Mobile Si substances represent monosilicic acid, polysilicic acid, organosilicon compounds and complex compounds with organic and inorganic substances (Matichenkov et al., 2001).

Beginning in 1848, numerous laboratory, greenhouse and field experiments have shown benefits of silicon fertilization for rice, corn, wheat, barley, sugar cane and other crops and benefits for maintaining a sustainable agriculture. Silicon fertilization has a double effect on the soil-plant system. Firstly, improved plant Si nutrition reinforces plant protective properties against diseases, insect attack and unfavorable climatic conditions. Secondly, the soil treatment with Si biogeochemically active substances optimizes soil fertility through improved water, physical, and chemical soil properties and maintaining nutrients in a plant-available form.

The role of Si in the nutrition of plant species used in horticulture has not been well investigated in comparison to agricultural crops like rice or sugarcane. Some authors have demonstrated the relevant uptake of this element for some plant species used in horticulture. Si supplements have been used for production of greenhouse crops in Europe. Now some growers and researchers consider Si as a 'quasi-essential' element for plant growth and development.

2. Si IN PLANTS

Plant absorbs Si from the soil solution in the form of monosilicic acid also called orthosilicic acid [H_4SiO_4] (Lewin and Reimann, 1969; Yoshida, 1975). Tissue analyses from a wide variety of plants found Si concentrations to range from 0.1% to 10% of dry weights depending on plant species (Epstein, 1999). Comparison of these values with those for such elements as P, N, Ca, and others shows Si to be present in amounts equivalent to those of macronutrients (Figure 1).

In plant, Si transports from root to shoot with the transportation steam in xylem. Xylem Si concentration has frequently been found to be very high (Savant et al., 1997). Silicon is concentrated in epidermal tissue. Monosilicic acid accumulated transforms to polysilicic acid and amorphous silica that can associate with pectin

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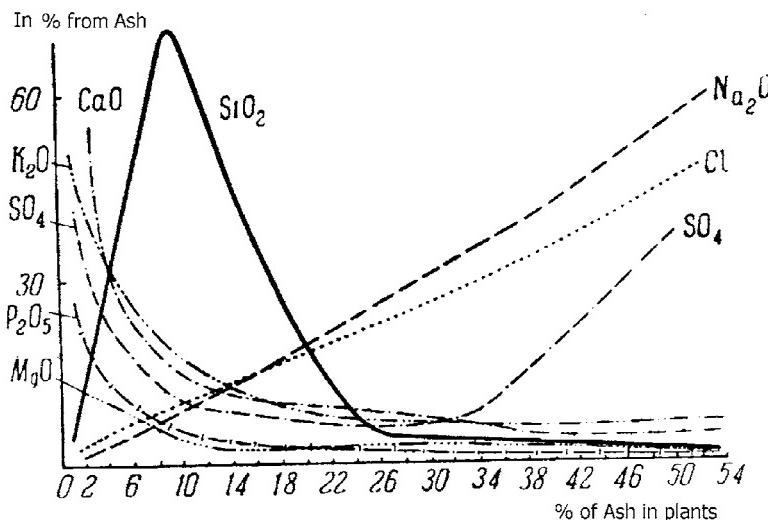
Figure 1. Si in ash of cultivated plants (Kovda, 1956).

and calcium ion (Waterkeyn et al., 1982). By this means, the double cuticular layer forms protecting and mechanically strengthening plants (Figure 2).

Silicon deposits in cell walls of xylem vessels prevent compression of the vessels under condition of high transpiration caused by drought or heat stress (Emadian and Newton, 1989). Si may alleviate salt stress in higher plants (Liang, 1999; Matichenkov et al., 2001). The below described interaction between monosilicic acid and heavy metals, Al, Mg can clarify the mechanism of reducing heavy metal, Mg and Al toxicity for plants by Si (Barcelo et al., 1993; Foy, 1992).

Optimization of Si nutrition results in increasing weight and volume of roots, total and adsorbing surfaces (Figure 3) (Adatia and Besford, 1986; Bocharnikova, 1996). Silicon fertilizer perfects root respiration (Yamaguchi et al., 1995). Silicon is assumed to have an effect on the fruit formation. The lack in Si nutrition has a negative effect on flowering and fruit formation (Miyake, 1993; Savant et al., 1997).

The negative effects of the lack in plant-available Si were detected for cucumber (*Cucumis sativus* L.) (Adatia and Besford, 1986), tomato (Miyake and Takahashi, 1978), strawberry (*Fragaria* spp.), black raspberry (*Rubus occidentalis* L.) (Lanning, 1960; Miyake and Takahashi, 1986), citrus (*Citrus* spp.) (Matichenkov et al., 1999, 2000; Taranovskaja, 1940, Wutscher, 1989).

3. EFFECT OF Si ON HORTICULTURAL PLANTS

Many authors have mentioned the effect of Si on enhancement of resistance against infections, diseases and insect attacks (Belanger et al., 1995; Voogt and Sonneveld, 2001). Soluble Si (potassium silicate) increased cucumber resistant against root diseases caused by *Pythium aphanidermatum* and by *Pythium ultimum* (Cherif et al., 1994; Cherif and Belanger, 1992). Optimization of Si nutrition increased plant
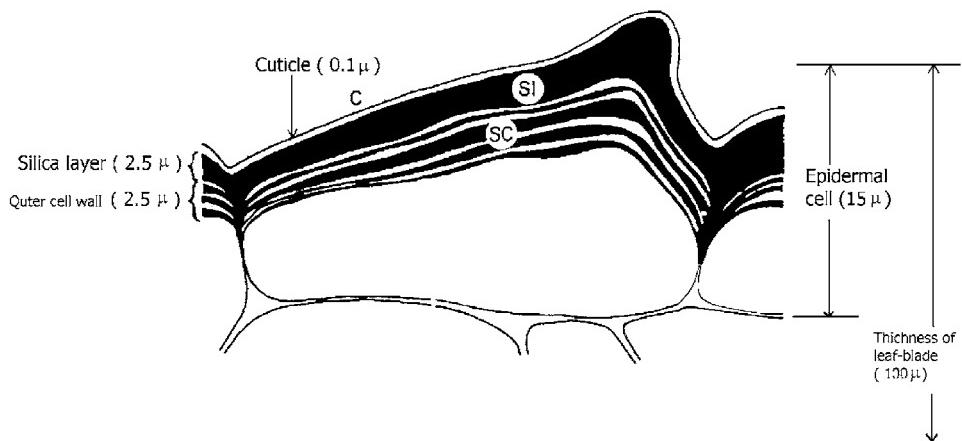
Figure 2. Schematic representation of the rice leaf epidermal cell (Yoshida, 1975).
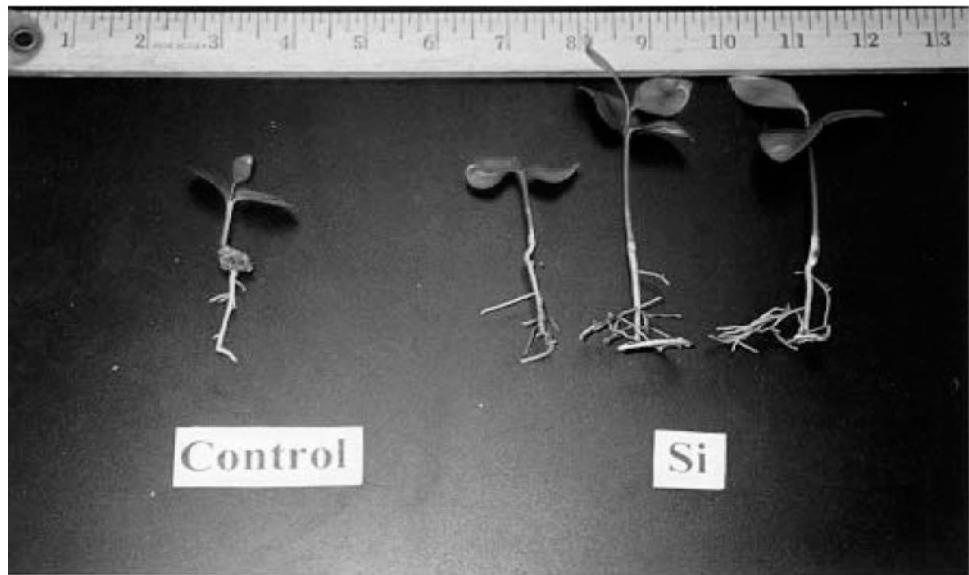
Figure 3. Effect of monosilicic acid on the root formation of germinated orange (Matichenkov et al., 1999).

protection against powdery mildew and other diseases and had greater yield response for cucumber and rose (*Rosa* spp.) grown on rockwool grown (Voogt, 1988, 1990, 1991, 1992).

3.1. Effect of Si on productivity of horticultural plants

The effect of Si fertilizers on horticultural plants was mostly investigated on cucumber and tomato (Table 1). Increased number of fruits and average fruit weight

Table 1. Effect of Si fertilizers on productivity of horticultural plants.

#	−Si			+Si			Si source
	Fruits, m^2	Kg, m^2	Fruit wt, g	Fruits, m^2	Kg, m^2	Fruit wt, g	
			Cucumber (Voogt and Sonneveld, 2001)				
1	85	326	384	87	334	384	Silica sol
2	74	324	438	85	361	425	waterglass
3	56	318	568	64	368	575	K$_2$SiO$_3$
4	18	82	456	21	109	519	K$_2$SiO$_3$
			Cucumber (Korndorfer and Lepsch, 2001)				
		Fruit yield, t ha^{-1}			Fruit yield, t ha^{-1}		
5		121			142		Ca-Si
6		121			155		K$_2$SiO$_3$
			Cucumber (Cherif et al., 1994)				
	Plant dry wt, g		Fruit per plant	Plant dry wt, g		Fruit per plant	
7	69.6		4.1	72.4		4.7	K$_2$SiO$_3$
8	136.7		2.8	257.1		5.0	K$_2$SiO$_3$
			Rose (Voogt and Sonneveld, 2001)				
	Stem, m^2	Stem wt, m^2	Kg, m^2	Stem, m^2	Stem wt, m^2	Kg, m^2	
9	144	40	5.8	159	38	6.0	K$_2$SiO$_3$
			Strawberry (Korndorfer and Lepsch, 2001)				
	Fruit per plot		Kg per plot	Fruit per plot		Kg per plot	
10	67.5		0.521	91.5		0.675	Ca-Si
			Tomato (Miyake, 1993)				
		Fruit wt, g			Fruit wt, g		
11		39.4			51.3		K$_2$SiO$_3$

have been observed with the Si application. During a period of 9 years, a number of Si experiments with cucumbers have been demonstrated that the yield increase differed from 6 to 16% for number of fruits and from 11 to 33% for total yield (Voogt and Sonneveld, 2001).

Y. Miyake (1993) demonstrated Si nutrition to be more important for tomatoes during first stage of plant growth and during flowering stage. The lack in plant-avail-

able Si had a negative effect on fruit formation process (Miyake and Takahashi, 1978).

The application of amorphous fine SiO_2 was responsible for increasing weight of plants and fruits in various potting mixtures (Table 2). First potting mixture was 5 years old and contained vermiculite, muck, surface horizon of Forest soil, and ceramic substances. Second potting mixture was 2 years old and contained sod, muck, and ceramic substances. Third potting mixture was 2 years old and contained sand with sod.

For tomatoes, improved Si nutrition resulted in increasing average weight of fruits from 243 to 274 and 289 g, respectively for 500 and 1000 kg ha^{-1} of SiO_2 (Table 2). Maximum effect of Si fertilization was examined in potting mixture 3. The average fruit weight increased from 68 g to 140 and 150 g, respectively for 500 and 1000 kg ha^{-1} of SiO_2 (Table 1).

3.2. Effect of Si fertilizers on citrus germination

Soluble Si has an effect on the plant germination (Diakov et al., 1990; Matichenkov, 1990). Citrus is considered as a non-Si accumulator (Wutscher, 1989). Therefore, citrus is convenient for demonstration of nonspecific effect of soluble Si on germination of plant seeds.

The experiment on orange (Volk) (*Citrus sinensis* L.x C.) seeds germination conducted with a commercial potting mixture for germination of citrus seeds ('Metro-Mix 500') showed that fine amorphous silica applied as a source of plant-available Si had a positive effect on the initial growth of orange seedlings (Table 3). The maximum mean dry shoot weight increased from 0.061 to 0.081 g when treated with

Table 2. The effect of amorphous silica on growth and productivity of tomatoes.

	Average height of plant, cm	Average number of fruits on 1 plant	Average weight of tomato fruit, g
potting mixture 1			
Control	85ab	10.1a	243b
SiO$_2$ 500 kg ha^{-1}	90a	9.4ab	274a
SiO$_2$ 1000 kg ha^{-1}	104a	10.3a	289a
potting mixture 2			
Control	63b	4.0c	40f
SiO$_2$ 500 kg ha^{-1}	70b	8.0b	70d
SiO$_2$ 1000 kg ha^{-1}	82ab	9.2ab	84d
potting mixture 3			
Control	30d	11.0a	68e
SiO$_2$ 500 kg ha^{-1}	40c	10.0a	140c
SiO$_2$ 1000 kg ha^{-1}	55c	12.4a	159c

Using Duncan's multiple range test, values within a column followed by the same letter are not statistically different ($P < 0.05$).

Si. The dry root weight increased from 0.062 to 0.070 g under amorphous silica application (Table 3).

3.3. Effect of Si on quality of horticultural plants

Silicon fertilizers have an effect on quality of horticultural products. High nitrate content in potting mixtures essentially increases the plant productivity but reduces plant resistance to diseases and quality of fruits (Matsuyama, 1975). Monosilicic acid was noted to be able to regulate nitrate absorption by plant (Litkevich, 1936; Mitsui, Takaton, 1963). If a soil is low in nitrates, the application of Si fertilizer increases plant nitrate concentration. On the other hand, if a soil contains nitrates in abundance, the optimization of Si nutrition results in reducing nitrate accumulation in fruits.

The application of activated silica to potting mixtures resulted in a significant decrease in nitrate (NO_3^-) content in the fruits of tomatoes (Table 4). The effect of Si fertilizer on the nitrates was maximal for potting mixture 1 rich in nitrates. The regulation of nitrogen concentration in plant tissue by Si fertilizer was demonstrated in other studies as well (Litkevich, 1936; Mitsui, Takaton, 1963).

Cherif M. with co-authors (1994) reported that application of high rates of phosphorus fertilizer resulted in 100% infection of cucumber by *Pythium aphanidermatum*. Using soluble Si together with phosphorus reduced the level of infection

Table 3. Effect of amorphous Si on the germinated oranges (Volk) in the potting mix 'Metro-Mix 500'.

Treatment	Fresh weight		Dry weight	
	Shoots	Roots	Shoots	Roots
	-- g --			
Control	0.301c	0.419c	0.061c	0.062b
SiO_2 0.5 g	0.298b	0.412c	0.071b	0.065ab
SiO_2 1 g	0.421a	0.523a	0.081a	0.070a
SiO_2 2 g	0.329b	0.446b	0.071b	0.062ab

Using Duncan's multiple range test, values within a column followed by the same letter are not statistically different ($P < 0.05$).

Table 4. The effect of amorphous silica on the nitrates in tomatoes, NO_3^-, mg kg^{-1}.

Potting mixes	Control	SiO_2
	------------------ NO_3^-, mg kg^{-1} soil ------------------	
1	98a	70b
2	83a	70b
3	56a	45b

Using Duncan's multiple range test, values within a raw followed by the same letter are not statistically different ($P < 0.05$).

by 60%. The mechanisms by which Si provides plant protection against pathogens, accelerates growth of plant roots and shoots, and increases quality of fruit remain debatable. The inability to decipher the Si biochemistry in plants has a severe impact on the horticultural industry by preventing the commercialization of Si-based products. Some greenhouse growers use Si fertilizers (King et al., 2000) but mainly growers are still waiting for an approval of Si fertilizer use by governmental agencies.

4. Si FERTILIZERS FOR HORTICULTURE

Characteristics of a satisfactory Si source are: a high content of soluble Si, physical properties conductive to mechanized treatment, ready availability, and reasonable cost. Many sources have been evaluated for use in horticulture. The purpose of application of a Si source is to provide soluble Si to plants; therefore a good source must have much of its Si readily soluble in the soil solution. Sodium and potassium silicates are used as liquid Si fertilizers for spraying or irrigation of horticultural plants (Mevzies et al., 2001, Voogh and Sonneveld, 2001). Usually, the concentration of monosilicic acid of 100 ml Si L^{-1} is suggested as optimum for dilution of liquid Si fertilizers. Liquid Si fertilizers are applied for acceleration of plant germination, propagation, and formation of fruits and for protection against diseases, infections and attack of insects. Liquid Si fertilizers have a short period of activity because of their high mobility.

Diatomaceous earth, silica aerogel, Si-rich minerals (montmorillonite, mica, wollastonite, tuff, volcanic ashes et al.), plant ashes, Si-rich slags may be used as solid Si fertilizers or soilless conditioners. Typical rates of the application of Si-rich materials by broadcasting or incorporated methods are from 1 to 4 tons ha^{-1}. Solid Si-rich substances are mixed with soilless media before seedling or propagation of plants. High rates of Si applicants provide 2–3 year complete Si nutrition for plants.

5. Si IN HORTICULTURAL MEDIA

Before the 1950s, horticultural growing media for containers consisted primarily of mineral soils. Mineral soils have many drawbacks for plants in containers including low air capacity, low water holding capacity, too much weight, and possible contamination by herbicides and other phytotoxic chemicals plus potential contamination by disease organisms. So, today many professional growers have switched to the soilless media or soilless media containing small percentage of soil or sand. Such media can be prepared from individual components such as sphagnum peat moss or bark. However, the mixes are generally prepared from materials listed below or similar. Common materials in use are sphagnum peat moss, hypnum peat, reed sedge peat, combusted bark, composted bark, fresh bark, composted organic wastes etc. Usually, these materials are poor in plant-available Si. In such soilless growing media systems, the Si contents in plant tissue were found to be significantly lower

in comparison with plants grown in a soil (Voogt and Sonneveld, 2001). So, to provide Si plant nutrition, the Si application to horticultural media could be beneficial.

Using of Si fertilizers requires the determination of the Si deficiency level in horticultural media. A good test should be simple, rapid, and perhaps duplicate in the laboratory as closely as possible the behavior of the nutrient extraction by plants. Various methods for determining plant-available Si in a growing media have been suggested. Usually, there are extraction methods from air-dry soil with using salt, acid or alkaline bearing solutions (Barbosa-Filno et al., 2001; Matichenkov, 1990). The evaluation of Si extraction methods is based on the coefficients of correlation between the soluble Si extracted from the growing media and Si in the plant tissue (Barbosa-Filno et al., 2001). For example, extraction with 0.5 M acetic acid provides good correlation with rice straw and panicle Si percentage ($r^2 = 0.899$) (Barbosa-Filno et al., 2001). Unfortunately, the content of only plant-available Si can't be determined on extracts from dry growing media. There are present various forms of soluble Si-rich substances: monosilicic acid, polysilicic acid, Si complexes with organic and inorganic molecules, organo-silicon compounds (Matichenkov, 1990). Plant absorbs only monosilicic acid that can be recognized as actual form of Si for plant nutrition. Drying samples results to polymerization and dehydration of all soluble Si-rich substances (Matichenkov et al., 1997). The restoration of the equilibrium between various Si-rich substances requires 2–3 week incubation with water (Savant et al., 1997). As a result, the concentration of Si in an extract from dried soil reflects the total content of soluble Si in growing media, but not plant-available or actual Si.

The concentration of absorbed monosilicic acid is restored fast by dissolving Si-rich materials such as fine amorphous silica, Si-rich finely dispersed minerals or phytoliths (Iler, 1979). The Si-rich materials with a high speed of dissolving represent the sources of plant-available Si or potential Si. Both actual and potential forms are important for successful Si plant nutrition and should be tested for determining Si deficiency in a growing media.

We suggest the evaluation of Si deficiency in a soil or horticultural media using water and acid extraction methods. The water extraction from fresh soil allows direct determination of plant-available Si or actual Si in a soil or horticultural media (Matichenkov et al., 1997). This method provides close correlations with Si in plant tissue ($r^2 = 0.96$) (Matichenkov et al., 1997). The results of the acid extraction by 0.1 n HCl from dry soil or soilless media are in a good agreement with the rate of Si fertilizer applied (Barsykova and Rochev, 1979). The method provides a close correlation with Si in plant tissue as well (($r^2 = 0.98$) (Matichenkov et al., 1997). The hydrochloric acid (0.1 n) partly dissolves amorphous Si and some Si-rich minerals that are the main sources for monosilicic acid in soil solution. Silicon analyzed on this extract may be identified as a potential Si. Both forms of Si (actual and potential) provide complete information about the real content of plant-available Si in horticultural media and its dynamic as a result of Si-rich material application.

5.1. Actual Si in horticultural media

The content of monosilicic acid or actual Si in a soil or a soilless media is analyzed by the following procedure. Fresh potting mixture or soil sample is collected and is kept in a field moisture condition after removing plant roots and passing through a 2 mm sieve. Six (6) g of soil are placed into 100-mL plastic vessels. Thirty ml of water are added to vessel. After 1 h shaking, a sample is filtered, and a clear extract is immediately analyzed for soluble monosilicic acid by Mallen and Raily method (Iler, 1979). Using this method has shown that a change in soil moisture from 6 to 50% has no effect on the concentration of soil soluble silicic acids which apparently are mobile and weakly adsorbed (Matichenkov et al., 1997; Matichenkov and Snyder, 1996). Soluble P also doesn't affect the determination of monosilicic acid.

This parameter is sufficient for controlling Si in horticultural media if liquid forms of Si applicants are used. In a case of solid Si fertilizers or Si-rich substances, determining actual and potential forms of mobile Si is profitable.

5.2. Potential Si in horticultural media

The content of acid-extractable or potential Si in a soil or a soilless media is deter-mined by the following procedure. Sample is air-dried and ground to pass through a 1 mm sieve. Two (2) g of soil are placed into 100 mL plastic vessels. 20-ml of 0.1 n HCl are added to vessel. After 1 h shaking, a sample is filtered, and a clean extract is analyzed for soluble monosilicic acid by Mallen and Raily method (Iler, 1979).

The following classification of deficiency of plant-available Si in soil or horti-cultural media is recommended (Table 5).

6. CONCLUSION

Now that we are aware of the role that Si plays in plant health and nutrition, we should no longer ignore its value. Mobile Si should be considered in any plant nutri-tion program and may provide an environmentally friendly tool for addressing problems with plant health, fruit quality and crop yield due to stresses and diseases related to tissue strength and rigidity. The suggested classification and methods

Table 5. Soil classification of deficiency of activated Si.

Level of deficiency	Actual Si	Potential Si
	--------------- mg kg^{-1} of Si in soil ---------------	
Without deficiency	>40	>600
Low level of deficiency	20–40	300–600
Deficiency	10–20	100–300
Critical deficiency	0–10	0–100

for determining actual and potential Si allow the evaluation of providing plant-available Si in a growing media. Today market of Si fertilizers suggests soluble and solid forms of Si applicants. However, numerous questions related with the role and functions of Si in plant and practical implication of Si-rich substances are still open.

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BIOLOGICAL MONITORING OF EXPOSURE TO PESTICIDES IN THE GENERAL POPULATION (NON OCCUPATIONALLY EXPOSED TO PESTICIDES)

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1. INTRODUCTION

Deliberate input of pesticides into the environment to exploit their toxic effects on antieconomic forms of life creates real possibilities of exposure beyond the occupational sphere. Once they have been introduced into the environment, currently used pesticides are relatively labile and tend not to persist for long. However, their widespread use makes it almost impossible for the average person to avoid exposure to low levels in his or her daily life (Morgan, 1992).

It is widely believed that low levels of exposure to current pesticides do not have acute toxic effects, however various types of cancer have been associated with chronic exposure to various groups of pesticides, such as the triazines and phenoxyacetic herbicides (Blair, 1990; Blair and Zahm, 1990; Zahm and Blair, 1992). Chronic exposure to pesticides has also been associated with effects on reproduction and various types of malformations in newborns (Sever et al., 1997; Tilson, 1998).

Well designed research to assess exposure of the general population to pesticides is therefore a fundamental necessity. For such studies to be accurate, they must be based on determination of individual exposure. Since exposure of the general population may occur through all environmental compartments and all routes of penetration, environmental monitoring of exposure should be respiratory and cutaneous. The assessment should be associated with analysis of pesticide residues in food actually ingested by the population. However, even considering all routes of penetration, the external dose may not accurately reflect the dose absorbed, which can only be determined by biological monitoring.

Assay of biological indicators of exposure has shown the constant presence of small quantities of widely used pesticides and pesticides that have become ubiquitous, or their metabolites, in biological fluids of the general, not occupationally exposed population. This finding suggests the importance of extending biological monitoring to the general population to systematically obtain biological reference values (BRV), at least for the more widely used compounds.

A series of limitations including gaps in our knowledge of toxicokinetics, metabolism and toxicodynamics of many pesticides, a shortage of validated analytical methods, the critical nature of the timing of placement of sample collectors and highly variable exposure modes have prevented large scale biological monitoring of exposure to pesticides. These limitations have also affected the definition of BRV and been made worse by factors such as sensitivity of analytical methods,

choice of study population and actual differences in environmental pollution in areas monitored, which may also depend on season.

Despite these problems, studies to define BRV of various pesticides have been undertaken at national and international level. The values obtained have different validity, depending on observation of a series of factors regarding collection and preparation of samples, analysis, choice of population, data processing and form of results.

2. SOURCES OF EXPOSURE OF THE GENERAL POPULATION TO PESTICIDES

The general population is mainly exposed to pesticides through:

– residues in food;
– proximity of residential areas to treatment areas;
– household use of pesticides.

In these three cases exposure is oral dietary; cutaneous and respiratory; cutaneous, respiratory and oral non dietary.

With regard to residues in food, a recent report of the Istituto Superiore di Sanità (Ministero della Sanità, 1998) demonstrates that in 7085 specimens of vegetables (44.7), fruit (51.9) and cereals (3.4) analysed in 1997 by Italian National Health Service laboratories, 78% were without residues (residue concentrations were below analytical detection limits, generally in the range 0.01–0.1 mg/kg), 21% contained residues within legal limits and the other 1% were above legal limits. Fruit was the most frequently contaminated product (29.2% of samples with residues within legal limits and 0.8% over the limit), followed by cereals (17.2% and 0.4% respectively) and vegetables (12% and 1.2% respectively). The most frequently contaminated products were celery, lettuce, escarole, spinach, chicory, apricots, kiwis, lemons, grapes, mandarins and wheat. The pesticides most frequently found above legal limits were chlorothalonil in vegetables, vinclozolin, acephate and carbendazim in fruit and pirimiphos-methyl in cereals. It seems likely that the percentage of specimens containing residues would rise considerably if the detection limits of the analytical procedures used improved by an order of magnitude, to be in line with those of the methods used for biological fluids (see below). Better knowledge of the distribution of residues in food, including non plant products (eggs, meat, dairy products) would be useful for evaluating daily dietary intake of the general population and to solve problems of toxicity associated with multiple exposure (simultaneous exposure to various pesticides).

The problem of food contamination with pesticide residues changes when supply is not through medium to large distribution for which suspension times before harvest are more or less observed, but through small local production or personal vegetable gardens. In the second case, there may be two opposite situations, namely production without use of pesticides or with incorrect use of pesticides. Other situations that may lead to elevated unrecognised contamination can be associated with treatment of nearby areas or aerial spraying (Aprea et al., 1996b).

Use of pesticides on a hobby basis in family vegetable gardens or gardens of houses, or outside to control pests, comes under the heading of proximity of residential quarters and treated areas. It results in exposure of the person using the pesticide, as in occupational exposure, and in exposure of members of the family. Domestic animals or the shoes of family members may bring contaminated dust into the house where pesticides break down more slowly (less exposure to sunlight, few microorganisms, low humidity) and may build up.

Another source of domestic use of pesticides is to combat parasites on ornamental plants and domestic animals. Domestic use is particularly widespread in the USA where an estimated 90% of families use these products indoors, particularly chlorpyrifos and diazinone (Gurunathan et al., 1998; Lebowitz et al., 1995; Robertson et al., 1999; Gordon et al., 1999). Shampoo against lice is not infrequent in school age children and consequently other members of the family. Cut flowers in houses are also another source of exposure due to the residues of treatments carried out before cutting.

For families in which one or more persons work with pesticides (agriculture, greenhouse work, chemical industry or preparation of formulas, spraying of public areas), para-occupational exposure of other members of the family due to contact with contaminated clothes, shoes or skin may occur. Work clothes can contaminate other clothes if washed together.

In studies for the definition of BRV, it is important to know the diffusion characteristics of the xenobiotics being monitored; if a substance is dispersed in homes largely as vapour, exposure of adults and children would be similar, whereas children are usually more exposed to substances dispersed as particulate because they play on the floor and put their hands in their mouths. Studies by EPA have shown that exposure to dusts deposited in homes is 12 times greater for children than adults (Lewis, 1989). Another important thing to consider is the route of absorption: if prevalently dietary, children are exposed more than adults, since children eat more food per unit body weight.

All sources of exposure to pesticides should be treated in the questionnaire given to subjects recruited for definition of BRV.

3. PROBLEMS CONNECTED WITH ESTIMATE OF BRV OF PESTICIDES

There are few published studies on the definition of reference values for pesticides, one reason being the problems connected with their determination which is different from the procedures used for other xenobiotics used in industry.

3.1. Toxicokinetics

The main advantage of biological monitoring is that it provides data that reflects the dose of xenobiotic taken up by the body via all routes of penetration.

Internal dose levels of pesticides vary with the entity and duration of exposure, as well as with their physicochemical nature and associated metabolic processes.

To guarantee quantitative data it is however necessary to know the toxicokinetics (metabolism, half-life, absorption and elimination) of the substance sufficiently well, preferably in humans. By virtue of their chemical nature, currently used pesticides have biological half-lives of only a few days, much briefer than toxic substances such as PCBs and dioxins which have half-lives of the order of years. Currently used compounds (e.g. carbamates, phosphoric esters, pyrethroids, triazines) generally do not remain in the body for long and do not tend to accumulate in tissues; they are metabolised rapidly to more polar compounds which are generally excreted in the urine. The variability of half-lives, which obviously depend on the chemical nature of the compound, must be considered together with biological variability which much be taken into account not only when adults are compared with children, but also for groups of the same age or in the same subject at different times. Metabolic variations depend on many factors (genetic, age, organ function) and the metabolism of a single substance may be affected by simultaneous exposure to other substances. However, little is known about interactions between different active ingredients during combined exposure. For example, organophosphate insecticides seem to inhibit hydrolysis and hence detoxification of pyrethroids. Some authors (Zhang et al., 1991) have reported higher urinary excretion of phenvalerate and deltamethrin in subjects using them combined with methamidophos, than in subjects using only the pyrethroids.

This complexity means that biological monitoring of exposure to pesticides is really very difficult and the interval between biological sampling and exposure is critical for defining internal dose.

Except for a few active principles, little is known about biological monitoring of human exposure; this is due to inadequate knowledge of toxicokinetics and metabolism of many compounds based on a lack of data to construct dose-response curves or to define levels of acute and long-term toxic effect. The only substance for which biological limits have been established are cholinesterase inhibitors (ACGIH, 2001; WHO, 1982; DFG, 1993), dinitro-o-cresol (WHO, 1982), lindane (WHO, 1982; DFG, 1993), parathion (ACGIH, 2001; DFG, 1993) and PCP (ACGIH, 2001; DFG, 1993). Hence proposed biological indicators are in most cases indexes of exposure and seldom indexes of internal dose.

These limits have reduced large-scale application of biological monitoring of occupational exposure to pesticides and hence the definition of BRV for the general population.

3.2. Analytical and preanalytical factors

Analytical procedures used to establish reference values need to be properly validated. Few such methods exist even for biological monitoring of occupational exposure.

Application of the method is subject to limits of detection (LOD): for determinations in urine of the general population, LODs of 1 µg/g or less are required, whereas LODs an order of magnitude higher are sufficient for biological monitoring of occupationally exposed subjects. For example, HPLC determination of phenoxyacetic herbicides (2,4-D, MCPA) with a LOD of 15 µg/g can only be used

for occupational exposure (Aprea et al., 1997b), whereas HRGC-ECD determination after derivatization of samples is sensitive enough for the general population (exposure (Aprea et al., 1997b; Hill et al., 1995; Holler et al., 1989). Similarly, the only available procedure to determine BRV of 2-thiazolidinethione-4-carboxylic acid (TTCA) has a LOD of 0.7 µg/g, involves derivatization of samples with diazoethane and uses HRGC/MS-SIM (Weiss et al.1999).

The methods of analysis used are generally complex and involve extraction, derivatization and purification of samples which normally gives precisions (percentage coefficient of variation CV%) greater than 10% (Aprea et al., 1996a). Accuracy is difficult to evaluate as no certified reference materials are available and the only procedure that can be used is interlaboratory comparison, after which sample concentration is defined by consensus (Aprea et al., 1996b; Aprea et al., 1999a). To increase accuracy, precision, sensitivity and specificity of analytical procedures, isotopic dilution is increasingly used. The stable isotopes used for this purpose have chemical and chromatographic behaviour practically identical to the analyte but the two can be distinguished during analysis on the basis of mass. Recovery is automatically corrected and the reduction of analytical variability is associated with a big reduction in LOD. As well, the bound compound acts as absolute reference of retention time, making mass spectrometry even more specific (Hill et al., 1995; Holler et al., 1989).

Analytical procedures used to define reference values sometimes involve assay of a metabolite (Aprea et al., 1999a, Aprea et al., 1993; Treble and Thompson, 1996) or group of similar metabolites (Aprea et al., 1997b; Aprea et al., 1996a; Hardt and Angerer, 2000). In these cases, the method is relatively simple and analytical reliability is easier to check. However, because of the cost, analysis time and quantity of matrix available, some studies require extraction of many analytes using the same analytical procedure (Hill et al., 1995; Holler et al., 1989; Shafik et al., 1973). In these cases, because of the different chemical structure of the analytes, selective isolation of single components cannot be done, which means that some metabolites are recovered much less efficiently in multiresidue methods and the analytical results must be corrected using suitable internal standards or stable isotopes.

This, together with the fact that results of samples from the general population are often close to the LOD, make determination of BRV anything but easy.

Preanalytical factors are also important in the definition of accurate reference values because it is necessary to ensure that the sample does not deteriorate or become contaminated between sampling and analysis. The most suitable sampling and conservation procedures should be defined for each analyte or group of analytes by special tests. Samples should be protected from the light: shielding is important in certain cases, for example ethylenethiourea breaks down to ethyleneurea on contact with light and certain activators such as chlorophyll and organic solvents (Ross and Crosby, 1973). In all other cases, shielding is a precaution (Aprea et al., 1999a). Preservatives and stabilising agents are not normally used because they could affect analysis. Samples are usually frozen at once and stored at –18/30 °C until analysis (Aprea et al., 1996b; Aprea et al., 1999a).

Since analysis is rarely carried out immediately after sampling, it is advisable to test for conservation: stability studies for 3,5,6-trichloro-2-pyridinol in urine

samples failed to detect any deterioration after 40 days at –18 °C (Aprea et al., 1999a). Under the same storage conditions, no significant breakdown of ethyl-enethiourea occurred in urine after 350 days (Aprea et al., 1996b). Alkylphosphates are stable in frozen urine for at least 20 weeks (Ito et al., 1979); another stability study showed that conjugated 2-isopropoxyphenol is stable in urine for at least 6 months at –20 °C (Leenheers et al., 1992).

4. USE AND UTILITY OF PESTICIDE BRVs

Determination of BRVs of pesticides is particularly important, especially for accurate assessment of occupational exposure levels. Since dose/response equations are unavailable for most compounds, and since few biological limits are therefore known, the available biological indicators are mainly indices of exposure and BRVs are thresholds above which a particular occupation is associated with exposure greater than that of the general population. Since BRVs express the contribution that a particular life-style makes to the biological indicators, they are therefore a target to aim for to limit the additional risk associated with certain occupational activities, over and above that to which the general population is exposed. This is why reference values are so useful for identifying emergency situations arising from poisoning or accidents due to illegal use of pesticides in the domestic environment (Hill et al., 1996).

BRVs make it possible to map the type and entity of contamination of the life environment: for example, chlorinated compounds such as DDT were used widely in the fifties and sixties and limited in the seventies. Some authors estimate that DDE concentrations in fatty tissue of the general population increase with the age of the population with a trend of about 7 ng/year; the trend of DDT is reported to be 0.9 ng/year (Gallelli and Mangini, 1995).

For many epidemiological studies, the availability of BRVs would also be a much more valid and direct support than values deduced from historical data or deduced subjectively or indirectly from pesticide residues in food samples.

5. CHOICE OF MATRIX

Biological monitoring involves measurement of xenobiotics and/or their metabolites in blood, urine and other biological materials. Due to the difficulty of obtaining tissue samples (invasive sampling) and the obvious limits of sampling saliva, sweat, expired air, feces and so forth (small quantities of material, sampling difficulty, low residue concentrations), available studies have largely been performed with urine, or sometimes blood. Blood has some advantages: the determination generally regards the compound itself rather than metabolites, so it is not necessary to have detailed information on metabolism. Blood volume does not vary when liquids are drunk or with other factors, so that concentrations of the toxic substance remain constant if the quantity absorbed is constant, and unlike for urine, corrections are not required for dilution. Concentrations of xenobiotics in blood often

peak immediately after exposure, making the time of sampling much less critical than for urine. However, concentrations of substance in blood may vary in relation to the route of absorption: ingested contaminants take longer to go into circulation than cutaneous or respiratory input. Compounds assayed in blood reflect the doses available to target organs more directly, since they have not yet been eliminated from the body. The main disadvantages of blood are linked to invasive sampling which limits samples from children and participation in large scale studies, and the usually low concentrations of compounds in blood. Quantities of blood available for analysis are small, and extremely sensitive analytical techniques, with LOD of the order of ng/l, are required.

Ease of sampling, a particular advantage when multiple samples are required or when biological monitoring is carried out in children, as well as the quantity of sample available for analysis, make urine the most widely used biological matrix in biological monitoring studies, especially large-scale studies on the general population. Analysis of urine is also facilitated by higher concentrations of toxic substances than in blood, due to their rapid metabolisation and excretion. However, the increase in the quantity of sample treated increases analytical interference due to the matrix. The main problem related to the choice of urine as matrix is the need to have information on metabolism of the substance. Very little information of this kind is available for pesticides and when it is available, it usually regards studies in experimental animals which do not necessarily extrapolate to humans. If metabolic data obtained in animal studies is used to develop an analytical method, it may lead to non detection of the metabolite in humans and to wrong conclusions that the dose of toxic substance absorbed is low (Driskell and Hill, 1997).

In most cases, BRV is determined by spot urine samples or sometimes from blood. Due to contingent difficulties, 24-hour urine samples are used in few studies (Treble and Thompson, 1996), although they are the only strategy for evaluating daily excretion of substances. Concentrations in spot samples are then normalised with respect to creatinine or urine specific weight. These methods of correction do not necessarily correct dilution of the sample because the speed of reabsorption of the metabolite in the renal tubules may be significantly different from that of creatinine; urine samples with creatinine concentrations below 0.30 g/l are regarded as too dilute for accurate correction (Lauwerys and Hoet, 1993).

The choice of matrix to analyse becomes more complex when a single metabolite may come from different compounds. For example, 1-naphthol is a human metabolite of naphthalene and carbaryl (Bienick, 1994; Knaak et al., 1968). In these cases, other information can be used to identify the source of exposure: when 1-naphthol is derived largely from naphthalene, its urinary excretion shows a correlation with that of 2-naphthol, another metabolite of naphthalene (Hill et al., 1995b). If exposure is also due to carbaryl, there is little correlation and the best choice is to assay 1-naphthol in serum as well, as naphthalene is metabolised much more slowly than carbaryl, and high serum concentrations usually reflect exposure to the pesticide rather than to naphthalene (Hill et al., 1995b).

The timing of sampling in the 24-hour period of a day may be important in defining reference values because it should be related to the kinetics of the compound studied. In some cases the second micturition of the day has been chosen (Aprea

et al., 1996b; Aprea et al., 1999a) to avoid the concentrated urine of the night and urine excreted after the main meals which are less likely to reflect the subject's body burden.

6. CHOICE OF STUDY POPULATION, EXCLUSION FACTORS AND STUDY OF VARIABLES

The sources of exposure of the general population to pesticides (mentioned above) are extremely variegated; this means that variability of results from biological samples is expected to be very high and may have outliers that are not always explained by the questionnaire used.

The relative homogeneity of food supply, at least on a national level, should not give rise to appreciable differences between samples obtained in the different regions of Italy, except those linked to different culinary habits or luxury items (tobacco, wine, spirits) which can, however, be detected by means of the questionnaire. In theory, the season in which sampling is done should not greatly affect the urban population as much as populations living near farming areas where pesticides are used. Seasonal differences in diet and luxury consumption should be detected by the questionnaire.

The number of samples must be sufficient to stratify the variables, some of which are common to other classes of compounds, and others of which are specific for pesticides. In the first group we have sex, age, medication, alcohol and smoking habits, all variables which may influence the metabolism of xenobiotics in general. In the second group we have all the factors already mentioned that may cause specific exposure to these substances. It is not unusual that variables like sex and age turn out to be significant because of habits associated with them (wine consumption, type and quantity of food eaten) (Aprea et al., 1996b; Aprea et al., 1996c). Wine consumption may also be a significant factor, not because of the alcohol but because of the pesticide residues it contains (Aprea et al., 1997a; Aprea et al., 1996b; Aprea et al., 1999a). Likewise, smoking may be significant because of traces of pesticides in the tobacco (Aprea et al., 1996b).

Since so many variables may influence exposure to pesticides in the general population, exclusion factors are often used in order to reduce the size of the population to investigate. A factor used in several Italian studies was direct exposure to pesticides in the home and at work (Aprea et al., 1999a). Domestic use is difficult to evaluate by questionnaire because people normally do not know the active ingredients in the products they use to treat ornamental plants, pets, and so forth. Other exclusion factors regarded smoking habits, age and medication (Aprea et al., 1999a).

In published studies, the influence of these variables on reported values is not always evaluated. Over the years, BRVs may be subject to variations, sometimes striking, due to the evolution of analytical methods and changes in the type and quantity of active principles used (Hill et al., 1995b; Murphy et al., 1983; Kutz et al., 1992; Hill et al., 1989; CDC, 2002).

Two examples of questionnaire for adults and children, respectively, used in two Italian studies (Aprea et al., 1996c; Aprea et al., 2000), appear in Appendix 1 and 2.

7. EXPRESSION OF REFERENCE VALUES FOR PESTICIDES

In 1995 Hill et al. (Hill et al., 1995b) defined pesticide 'reference range concentrations' as biological concentrations of a specific metabolite expected in members of the general population, who have not had occupational exposure to the compounds.

In monitoring pesticides in the general population, the percentage of samples with concentrations above the LOD is hardly ever 100%. Because of the difficulty of statistical analysis of undetectable data, it is problematical to define BRVs by means of point values, such as arithmetic and geometric mean. If only consider data with concentrations above LOD are considered, our assessment will be an arbitrary overestimate. If we set undetectable levels equal to LOD or LOD divided by two, we are forcing the data, especially if more than 10% of samples are undetectable. In our opinion, the best way to express BRV is the 5–95 percentile range of the data of a given population. If the percentage of undetectable concentrations is less than 10%, BRV can be expressed as mean, using LOD divided by two for undetectable samples in the statistical analysis.

8. BIOMARKERS BEING STUDIED TO DEFINE BIOLOGICAL REFERENCE VALUES FOR PESTICIDES

Studies for the definition of biological reference values for pesticides have been underway for some time throughout the world, the aim being to verify the various systematic studies that have been conducted into residues in food and to achieve a better understanding of the impact of these products, deliberately introduced into the life environment, on humans.

Table 1 shows biomarkers evaluated in at least one of the studies published on populations not occupationally exposed to pesticides. The table also indicates the possible origin of these biological indicators, which are sometimes non specific and may be derived from compounds not used as pesticides.

Pesticides which undergo little or no transformation by the body have been determined unmodified in biological fluids. These measures have the advantage of high specificity and exist for cyclopentadiene organochlorines (aldrin, dieldrin, endrin, chlordane, heptachlor), derivatives of phenoxycarboxylic acids (2,4-D, 2,4,5-T, dicamba, silvex), pentachlorophenol and hexachlorobenzene also in samples of fat, plasma and serum (Gallelli and Mangini, 1995; Murphy et al., 1983; Pavan et al., 1987).

Table 1. Biomarkers evaluated in published studies for the definition of pesticide BRVs.

Country (Reference)	Biomarker (biologic matrix)	Possible origin[a]
USA (Hill et al., 1995; Murphy et al., 1983; Kutz et al., 1992)	Carbofuranphenol (urine) (CFF)	benfuracarb, carbofuran, carbosulfan, furathiocarb
USA (Hill et al., 1995b; Murphy et al., 1983)	1-Naphthol (urine) (1NAP)	carbaryl, naphthalene, napropamide
USA (Hill et al., 1995b; Murphy et al., 1983)	2-Isopropoxyphenol (urine) (IPP)	propoxur
USA (Murphy et al., 1983)	3-Ketocarbofuran (urine) (KCF)	carbofuran
Italy (Aprea et al., 1996c; Aprea et al., 2000); USA (Murphy et al., 1983; CDC, 2002); Germany (Hardt and Angerer, 2000)	Alkylphosphates (urine) (DMP, DMTP, DMDTP, DEP, DETP, DEDTP)[b]	Phosphoric esters
Italy (Aprea et al., 1999a); USA (Hill et al., 1995b; Murphy et al., 1983; Kutz et al., 1992)	3,5,6-Trichloro-2-pyridinol (urine) (TCP)	chlorpyrifos, chlorpyrifos-methyl
USA (Hill et al., 1995b; Murphy et al., 1983; Kutz et al., 1992)	para-Nitrophenol (urine) (PNP)	chlomitrofen, EPN, fluorodifen, methyl-parathion, 4-nitroanisole, nitrobenzene, nitrofen, parathion
USA (Murphy et al., 1983; Kutz et al., 1992)	-monocarboxylic (urine) (MCA) e dicarboxylic acid (urine) (DCA)	malathion
Italy (Aprea et al., 1997b); USA (Hill et al., 1995b; Murphy et al., 1983; Kutz et al., 1992; Hill et al., 1989)	2,4-Dichlorophenoxyacetic acid (urine) (24D)	2,4-D
USA (Murphy et al., 1983; Kutz et al., 1992)	Dicamba (urine)	dicamba
USA (Murphy et al., 1983; Kutz et al., 1992)	2,4,5-Trichlorophenoxyacetic acid (urine) (2,4,5-T)	2,4,5-T
USA (Murphy et al., 1983; Kutz et al., 1992)	Silvex (urine)	Silvex
USA (Hill et al., 1995b; Hill et al., 1989; Germany (Angerer et al., 1992a; Angerer et al., 1992b)	2,4-Dichlorophenol (urine) (24DCP)	bifenox, chlomethoxyfen, 2,4-D (precursor during synthesis), 2,4-DB, dichlofenthion, diclofop,1,3-dichlorobenzene, dichlorprop, nitrofen, phosdiphen, prothiofos
USA (Hill et al., 1995b)	2-Naphthol (urine) (2NAP)	naphthalene, naproanilide, (2-naphthyloxy)acetic acid
USA (Hill et al., 1995; Hill et al., 1989); Germany (Angerer et al., 1992a; Angerer et al., 1992b)	2,5-Dichlorophenol (urine) (25DCP)	p-dichlorobenzene

USA/Canada (Treble and Thompson, 1996; Hill et al., 1995b; Murphy et al., 1983; Hill et al., 1989) Germany (Angerer et al., 1992b)	Pentachlorophenol (urine) (PCP)	PCP
USA (Hill et al., 1995b; Kutz et al., 1992; Hill et al., 1989; Germany (Angerer et al., 1992a; Angerer et al., 1992b)	2,4,5-Trichlorophenol (urine) (245TCP)	fenchlophos, lindane, pentachloronitrobenzene, pentachlorophenol, 1,2,4-trichlorobenzene, trichloronat, 2,4,5 T (precursore during synthesis)
USA (Hill et al., 1995b; Hill et al., 1989); Germany (Angerer et al., 1992a; Angerer et al., 1992b)	2,4,6-Trichlorophenol (urine) (246TCP)	chlornitrofen, hexachlorobenzene, lindane, pentachloronitrobenzene, pentachlorophenol, prochloraz, 1,3,5-trichlorobenzene
USA (Hill et al., 1989)	2,6-Dichlorophenol (urine) (26DCP)	lindane
USA (Hill et al., 1989)	3,4-Dichlorophenol (urine) (34DCP)	1,2-diclorobenzene
Italy (Brugnone et al., 1993)	Carbon disulphide (urine and blood) (CS2)	dithiocarbamates, carbon disulphide
Germany (Weiss et al., 1999)	2-Thiazolidinethione-4-carboxylic acid (urine) (TTCA)	dithiocarbamates, captan, carbon disulphide
Italy (Aprea et al., 1997a; Aprea et al., 1996b)	Ethylenethiourea (urine) (ETU)	ethylenebisdithiocarbamates (EBDC)
USA (Murphy et al., 1983); Italy (Pavan et al., 1987); Germany (Angerer et al., 1992b)	Hexachlorobenzene isomers (fat, serum, plasma) (HCB)	HCB
USA (Murphy et al., 1983); Italy (Gallelli and Mangini, 1995; Pavan et al., 1987)	DDT (fat, serum)	DDT and similar
Italy (Gallelli and Mangini, 1995; Pavan et al., 1987)	Hexachlorocyclohexane (fat) (HCH)	HCH
USA (Murphy et al., 1983); Italy (Gallelli and Mangini, 1995; Pavan et al., 1987)	Aldrin, dieldrin, endrin (fat, serum)	aldrin, dieldrin, endrin
USA (Murphy et al., 1983); Italy (Gallelli and Mangini, 1995; Pavan et al., 1987)	Heptachlor, heptachloroepoxide, trans-nonachlor, oxichlordane (fat, serum)	chlordane, heptachlor
USA (Murphy et al., 1983)	Mirex (fat, serum)	mirex

[a] The list of possible compounds is based on available informations on metabolism in humans and animals, or deduced from pesticide structure and possible metabolism.

[b] (DMP = dimethylphosphate, DMTP = dimethylthiophosphate, DMDTP = dimethyldithiophosphate, DEP = diethylphosphate, DETP = diethylthiophosphate, DEDTP = diethyldithiophosphate).

9. AMERICAN STUDIES

9.1. Biological reference values

The first major study completed on the question of pesticide BRVs was the II US National Health and Nutrition Examination Survey (NHANES II) by the National Center for Health Statistics (NCHS) in collaboration with the Human Monitoring Program for Pesticides of the Environmental Protection Agency (EPA) from 1976 to 1980 in 64 areas, surveying about 20,000 people of different ages, social background and profession, who underwent clinical examination, blood chemistry and functional testing, including testing for pesticide residues in 4200 blood samples and about 6000 urine samples. The study also included analysis of 785 samples of fatty tissue obtained in 1978 under the National Human Adipose Tissue Monitoring Program (Murphy et al., 1983; Kutz et al., 1992).

From 1988 to 1994, a group of about 1000 adults, age 20–59 years, from different regions and enrolled in the III National Health and Nutrition Examination Survey (NHANES III) gave urine samples which were analysed for 12 possible derivatives of pesticides (Hill et al., 1995b; NCHS, 1994).

In the same period, a study was conducted to determine 12 analytes (chlorphenols and phenoxyacetic herbicides) in urine of about 200 children, age 2–6 years, living in Arkansas (Hill et al., 1989). The aim of the study was to evaluate exposure of a group of about 100 children living near a herbicide factory and compare it with exposure of an age-matched group of children in a control community. Since no statistically significant differences were found, the authors combined the results for use as reference values for future studies.

The design and strategy of these three studies are shown in Table 2. The analytical procedures and parameters are shown in Table 3.

An important aspect of these studies was analytical quality control. In NHANES II, samples spiked with known quantities of the compounds of interest were analysed by the two participating laboratories. The samples were then used as internal quality control for 6 months (control cards). About 20% of the samples analysed by the method of Shafik et al. (1973) were selected randomly and analysed for confirmation using GC with Hall detector (electrolytic conductivity detector in halogen mode). If confirmation was not obtained, the data was included as not detectable. All determinations that were positive with the method of Bradway and Shafik (1977) were confirmed by reanalysis of samples.

Table 4 shows the results of three American studies on urine samples (Hill et al., 1995b; Murphy et al., 1983; Kutz et al., 1992) together with a blood analyte (p-DCB) assayed in NHANES II (Hill et al., 1995c).

The results of statistical analysis of this data afforded some surprises and induced the authors to draw some well founded conclusions about the widespread nature of exposure to pesticides in the general population and about a series of problems, already mentioned here, concerning the definition of BRVs for these substances. We shall now briefly look at the analytes, beginning with compounds most frequently detected, namely those with a high percentage of positivity (%pos).

A metabolite of p-dichlorobenzene, 25DCP, was detected with maximum con-

Table 2. Design and strategy of American studies for the determination of BRVs

	NHANES II (Murphy et al., 1983)	NHANES III (Kutz et al., 1992)	Arkansas children (Hill et al., 1989)
No. samples	6990	1000	200
Age of population (years)	12–74	20–59	2–6
Sample type	urine, blood and fat	urine	first morning urine
Sample conservation	freezing		freezing (–20 °C)
Quality control/Quality assurance	control cards	control cards	Quality controls in analytical series
Sampling period	1976–1980	1988–1994	–
Sample containers	20 ml glass containers shielded from light; no preservatives or stabilizers added	–	–
Analysis	done by two labs both using methods of Shafik et al. (1973) and Bradway and Shafik (1977)	method of Hill et al. (1995a)	method of Holler et al. (1989)
Sampling design	non random. Calculation of sample weights (different probability of selection) to obtain correct population estimates.		children living near herbicide factory compared with control population
Statistic analysis	calculation of %pos*. Because of asymmetrical distribution the data was log trasformed and expressed as geometric mean and 95% confidence interval.	calculation of %pos*, distribution percentiles and reference range concentrations	calculation of %pos * and distribution percentiles

* Percentage of samples with concentration greater than LOD.

Table 3. Analytical procedures used in American studies.

	Shafik et al. (1973)	Bradway and Shafik (1977)	Hill et al. (1995a)	Holler et al. (1989)
Analytes	dicamba, silvex, 245TCP, TCP, 4NP, 245T, 24D, PCP	MCA and DCA	IPP, 25DCP, 24DCP, CFF, 246TCP, TCP, 4NP, 245TCP, 1NAP, 2NAP, 24D, PCP	26DCP, 34DCP, 25DCP, 24DCP, 24D, PCP, 245TCP, 246TCP
Urine volume (ml)	1–5		10	10
Hydrolysis	Acid	Acid	Enzymatic (β-glucuronidase-aryl sulfatase)	Acid
Analyte isolation	Ether extraction	Extraction with ether/acetonitrile	Extraction with 1-chlorobutane/ether	Benzene extraction
Derivatization	Diazoetano	Diazomethane	1-chloro-3-iodopropane	Diazoethane
Purification	Silica ge	Silica gel	SPE (silica)	Silica gel
Apparatus	GC-ECD	GC-FPD	GC/MS/MS PCI (NCI for PCP) (isotopic dilution)	GC/MS/MS PCI (isotopic dilution or homologous internal standard)
LOD µg/l	5 µg/l (dicamba, silvex, 245TCP, TCP); 10 µg/l (4NP, 245T); 30 µg/l (24D); 2 µg/l (PCP)	30 µg/l for MCA DCA	1 µg/l for all analytes; 2 µg/l for 246TCP	1 µg/l for all analytes
% recovery	85–98%	Not reported	Accuracy +2/–6% (mean difference with respect to expected concentration)	33–164%
CV%	Not reported	Not reported	CV% between series 8.7–24	CV% between series 14–41

centrations of 12000 and 860 µg/l in 98% and 96% of samples analysed in NHANES III (Hill et al., 1995b) and in the Arkansas child study, respectively (Hill et al., 1989). Although the %pos were not dissimilar, values in children were much lower. This difference is probably due to the physicochemical characteristics of p-DCB, which being volatile, goes into the atmosphere. The ubiquitous nature of the parent compound in the life environment was demonstrated by studies conducted in the USA in 1987: it was found in 80% of houses tested and concentrations in personal air samples were 0.02–2600 µg/m3 (Wallace et al., 1987). IARC classifies p-DCB as possibly carcinogenic for humans (IARC, 1977). It is principally used in toilet deodorants and repellents as chemical intermediate for polymers. Occupational exposure is associated with urinary concentrations of 10,000–233,000 µg/l which is 13–300 times greater than 95% of the reference range established for adults (Pagnotto and Walkley, 1965). The results obtained in urine for 25DCP show a correlation with blood concentrations of p-DCB. Studies on the German popula-tion (248 samples analysed as control group to assess exposure of employees of a municipal waste incinerator) showed a %pos of 88% for 25DCP plus 24DCP with median, 95% and maximum of 3.93, 46.40 and 206.90 µg/g creat respectively (Angerer et al., 1992b).

Lower levels have also been found in children with respect to adults for 24DCP. The good correlation obtained by the authors with 25DCP partly demonstrates a common source, probably m-dichlorobenzene present as impurity in p-dichloroben-zene (Hill et al.1995b).

Sometimes residues analysed in biological fluids may have multiple sources, being linked to metabolic transformation of a number of compounds. An example is 1NAP, one of a series of compounds analysed in urine in NHANES III. This metabolite of carbaryl was found in urine of 86% of the subjects examined, often associated with 2NAP (81%). The good correlation between the two compounds obtained by the authors shows a common source, probably naphthalene (Bienick et al., 1994) a ubiquitous contaminant found in oil distillation products, mothballs and tobacco smoke. 1NAP is also used as a marker of exposure to polycyclic aromatic hydrocarbons (PAHs) (Hansen et al., 1994). The highest concentrations of 1NAP observed in NHANES III (maximum values about 25 times those of 2NAP) suggest a different source, perhaps carbaryl or PAHs in general. Occupational exposure to carbaryl is associated with urinary concentrations of 1NAP of 6200–78800 µg/l in industry and 70–1700 µg/l in agriculture (Shafik et al., 1971). These values are 2–2000 times 95% of the reference range.

Comparing the %pos for 1NAP of NHANES III and II, we are faced with com-pletely different values (86% versus 2%). This situation is common to other analytes determined in the two studies, for example 24D, PNP, 245TCP and TCP. The dif-ferences are partly due to different detection limits of the analytical procedures used, which were 5–30 times lower in NHANES III with respect to NHANES II for these analytes.

More can be said about TCP, a metabolite of chlorpyrifos and chlorpyrifos-methyl. This metabolite was detectable in 5.8% of samples in NHANES II and 82% of samples of NHANES III. The effect of the improved LOD (1 µg/l) of the method used in the latter study was a factor in this discrepancy but not the only one, because

Table 4. Results (µg/l) of American studies for the determination of BRVs in urine and blood samples.

Analyte	% Pos	N	Mean	5%	25%	50%	75%	90%	95%	99%	100%	Study (Reference)
CFF urine	1.5	902	<1	ND	ND	ND	ND	ND	ND	1.4	8.5	NHANES III (Hill et al., 1995b)
	4	6000	–	–	–	–	–	–	–	–	–	NHANES II (Kutz et al., 1992)
KCF	3	6000	–	–	–	–	–	–	–	–	–	NHANES II (Kutz et al., 1992)
24DCP urine	64	900	9.3	ND	ND	1.8	6.6	22	45	120	270	NHANES III (Hill et al., 1995b)
	27	197	–	–	ND	ND	1.0	–	11	–	110	Arkansas children (Hill et al., 1989)
25DCP urine	98	892	150	3.4	9.7	24	80	370	670	1800	12000	NHANES III (Hill et al., 1995b)
	96	197	–	–	5.0	11	25	–	200	–	860	Arkansas children (Hill et al., 1989)
24D urine	12	896	<1	ND	ND	ND	ND	1.2	1.5	5.1	9.6	NHANES III (Hill et al., 1995b)
	20	197	–	–	ND	ND	ND	–	2	–	12	Arkansas children (Hill et al., 1989)
	0.3	6990	–	–	–	–	–	–	–	–	212	NHANES II (Kutz et al., 1992)
IPP urine	6.8	902	<1	ND	ND	ND	ND	ND	1.6	4.3	9.6	NHANES III (Hill et al., 1995b)
	4	6000	–	–	–	–	–	–	–	–	–	NHANES II (Murphy et al., 198)
1NAP urine	86	891	15	ND	1.4	3.4	9.6	21	36	190	1400	NHANES III (Hill et al., 1995b)
	2	6000	–	–	–	–	–	–	–	–	–	NHANES II (Murphy et al., 1983)
2NAP urine	81	893	5.4	ND	1.1	2.6	7.6	14	18	32	48	NHANES III (Hill et al., 1995b)

Compound												Reference
PNP urine	41	886	1.2	ND	ND	ND	1.3	2.2	3.8	9.5	44	NHANES III (Hill et al., 1995b)
	2.4	6990	–	–	–	–	–	–	–	–	143	NHANES II (Kutz et al., 1992)
PCP urine	64	886	1.8	ND	ND	1.2	2.0	3.7	5.4	9.6	29	NHANES III (Hill et al., 1995b)
	100	197	–	–	8	14	59	–	160	–	330	Arkansas children (Hill et al., 1989)
	71.6	6990	–	–	–	6.0	–	15.5	–	–	2670	NHANES II (Kutz et al., 1992)*
245TCP urine	20	847	<1	ND	ND	ND	ND	1.4	2.0	6.1	19	NHANES III (Hill et al., 1995b)
	54	197	–	–	ND	1	2	–	7	–	30	Arkansas children (Hill et al., 1989)
	3.4	6990	–	–	–	–	–	–	–	–	5	NHANES II (Kutz et al., 1992)
246TCP urine	9.5	867	<2	ND	ND	ND	ND	ND	3.2	15	28	NHANES III (Hill et al., 1995b)
	11	197	–	–	ND	ND	ND	–	3	–	34	Arkansas children (Hill et al., 1989)
TCP urine	82	900	3.1	ND	1.3	2.2	3.5	6.3	8.3	16	34	NHANES III (Hill et al., 1995b)
	5.8	6990	–	–	–	–	–	–	–	–	104	NHANES II (Kutz et al., 1992)
26DCP urine	3	197	–	–	–	ND	ND	–	ND	–	7	Arkansas children (Hill et al., 1989)
34DCP urine	6	197	–	–	ND	ND	ND	–	1	–	9	Arkansas children (Hill et al., 1989)
Dicamba urine	1.4	6990	–	–	–	–	–	–	–	–	58	NHANES II (Kutz et al., 1992)
MCA urine	1.1	5973	–	–	–	–	–	–	–	–	970	NHANES II (Kutz et al., 1992)
DCA urine	0.5	5973	–	–	–	–	–	–	–	–	250	NHANES II (Kutz et al., 1992)
p-DCB blood	96	1000	2.1	–	–	0.33	–	4.8	11.0	–	49	NHANES II (Kutz et al., 1992)

* The authors (Kutz et al., 1992) report 10% (2.6 µg/l), geometric mean (6.3 µg/l) and 95% confidence interval of the geometric mean (5.9–6.6 µg/l).
2,4,5-T and silvex were analyzed in the same study but did not reach detection level

31% of the concentrations of NHANES III were above 5 µg/l, the LOD of the method used in NHANES II. Greater use of chlorpyrifos as domestic insecticide instead of the termiticide chlordane may be another explanation for the discrepancy, as figures on the utilisation of these products suggest.

The results of NHANES III for TCP are similar to those of an Italian study promoted by the Italian Society for Reference Values (ISRV) in 42 samples of the general population living in the towns of Pavia, Siena and Trento. The %pos found was 88%, with mean and maximum concentrations of 4.1 and 13.7 µg/l (Aprea et al., 1999a). The Italian levels were not influenced by domestic use of insecticides because the population selected had not had contact with any pesticide in the previous year. Factors significant for explaining variance of the data were consumption of wine and largely vegetarian diet.

In other cases, the use of analytical methods with lower LODs did not result in an increase in the frequency of positive samples. PCP, a compound widely used as disinfectant, but mainly as wood preservative, is an example. Use of PCP was restricted by EPA in 1984 (EPA, 1984). PCP exceeded the LOD of 2 µg/l in 71.6% of urine samples analysed in NHANES II and 64% of those analysed in NHANES III (LOD 1 µg/l), the urine samples of which were obtained in the period 1988–94, in other words, after the ban. The %pos was 100% in children of Arkansas with maximum and 95% levels being about ten times greater than those found in NHANES III. The higher urinary concentrations found in children were probably due to residues in food, which is consumed in greater quantities per unit body weight by children.

Various other studies on urinary concentrations of PCP are reported in the literature. One conducted in 1989 (Cline et al., 1989) with 143 Americans not occupationally exposed to the substance, found PCP in 100% of samples, with median and maximum of 3 and 17 µg/l, respectively. In subjects living in houses treated with PCP, concentrations were about 30 times higher than the reference range mentioned in NHANES III, with mean values of 69 µg/l.

In a subsequent German study on 248 urine samples analysed as control group to assess exposure of urban incinerator workers, all samples were positive and the mean, median, 95% and maximum concentrations of PCP were 3.2, 2.2, 8.7 and 67.7 µg/g creat, respectively (Angerer et al., 1992b). In a further study on 87 non occu-pationally exposed Canadians, all samples were positive and median and maximum concentrations were 1.3 and 9.1 µg/l, respectively (Thompson and Treble, 1994). Finally, a recent Canadian study in 1996 on 24-hour urine samples of 69 members of the general population showed a %pos of 94% with median and maximum con-centrations of 0.5 and 3.6 µg/l (Treble and Thompson, 1996). Use of 24-h urine samples made it possible to determine daily excretion of PCP, which averaged 1.1 µg, with median and maximum of 0.7 and 5.4 µg, respectively. The analytical method had a LOD of 0.05 µg/l and consisted of four steps: acid hydrolysis of 10 ml urine, extraction with petroleum ether, derivatisation with diazomethane and analysis by GC/MS-SIM with assay of isotopically labeled $^{13}C_6$PCP (Treble and Thompson, 1996).

For PNP, differences in the LOD of the analytical procedures used seem to explain the differences in %pos observed in the two NHANES studies. The %pos of 41%

observed in the more recent study with a LOD of 1 µg/l becomes 1.7% if we only consider values above 10 µg/l, the LOD of the method used in NHANES II. Hence for this analyte there do not seem to be differences in contamination levels over the years. According to the authors, the source of exposure was not parathion, EPN or nitrobenzene but a drug, acetaminophen, which seems to be synthesised from 4NP. In subjects exposed to parathion during industrial formulation, mean PNP concentrations were 900 µg/l and 4300 µg/l, depending on the precautions taken (Davies et al., 1966). In a case of fatal poisoning by parathion, concentrations of 40,300 µg/l were recorded, and in a non fatal case 10,800 µg/l (Davies et al., 1966).

Also for 24D, the difference in LOD of the procedures used in the two NHANES studies seems to explain the differences in %pos. In NHANES III, 12% of samples were positive, which drops to 0.1% if only measurements over 30 µg/l are considered. The %pos was slightly higher in children from Arkansas, supporting the hypothesis that the source of intake was residues in food. The 20% positivity found in the latter study is similar to that obtained in an Italian study (Aprea et al., 1997b) of 100 children, age 6–7 years: the maximum concentration observed was 2.5 µg/l, much less than that found in the American children. Concentrations of 24D found in occupationally exposed subjects vary widely according to exposure conditions (Aprea et al., 1995).

Concentrations of 245TCP and 246TCP obtained in NHANES III show a good correlation, suggesting some common sources of exposure, probably lindane, of which they are the main metabolites. The results of the Arkansas child study seem to indicate higher %pos for 245TCP. The reason may be lindane residues in food. Also for 245TCP, differences in LOD of the analytical procedures seem to almost completely explain the differences in %pos: the 20% of NHANES III drops to 2.2% if only levels above 5 µg/l are considered. The American data is not too different from that of the German study on 248 urine samples analysed as control group in an assessment of exposure of incinerator personnel: %pos was 54% for 245TCP and 37% for 246TCP with mean, median, 95% and maximum of 1.6, 0.8, 4.0 and 53.0 µg/g creat for 245TCP and 1.2, 0.6, 3.7 and 10.6 µg/g respectively for 246TCP (Angerer et al., 1992b). Studies on persons occupationally exposed to lindane revealed mean concentrations of 900 µg/l (Angerer et al., 1983; Pekari et al., 1991).

The very low %pos observed for the other analytes considered (IPP, CFF, KCF, dicamba, MCA, DCA, 26DCP, 34DCP, 245T and silvex) do not enable any useful conclusions to be drawn.

Table 5 shows the results of the American studies for determining BRVs on serum and fat samples (Murphy et al., 1983), compared with similar studies from the literature (Gallelli and Mangini, 1995; Pavan et al., 1987). The data indicates the existence of generalised exposure of the general population to certain organochlorine pesticides, such as total DDT, which was found in 99% of serum samples and 100% of fat samples analysed in NHANES II. For most other analytes, %pos in fat was much higher than in plasma, showing the distribution and accumulation of these substances in fat of the human body.

Table 5. Results of American studies for the determination of BRVs in serum and fat samples compared with similar studies from the literature.

	Matrix	No.	% Pos	GM	Mean ± SD	50%	95%	100%	Study (Reference)
HCB (*ppm*)	fat	92	58	–	0.31 ± 0.31	–	–	1.2	Italy (Pavan et al., 1987)
β-HCB	fat – serum	785–4200	93–4	–		–	–	–	NHANES II (Murphy et al., 1983)
HCB (other isomers)	fat – serum	785–4200	94–14	–	–	–	–	–	NHANES II (Murphy et al., 1983)
HCB (μg/l)	fat – serum	785–4200	<1–<1	–	–	–	–	–	NHANES II (Murphy et al., 1983)
	plasma	248	100	–	4.7	2.8	15.7	29.1	Germany (Angerer et al., 1996b)
HCH (*ppm*) Lindane (ppb)	fat	92	93	–	0.66 ± 0.54	–	–	2.6	Italy (Pavan et al., 1987)
	fat	28	96	68	104 ± 93.1	–	–	–	Italy (Gallelli and Mangini, 1995)
p,p′-DDE (ppm)	fat	92	100	–	1.11 ± 0.80	–	–	3.77	Italy (Pavan et al., 1987)
p,p′-DDT (ppm)	fat	92	100	–	0.12 ± 0.09	–	–	0.6	Italy (Pavan et al., 1987)
	fat	28	96	0.056	0.06 ± 0.03	–	–	–	Italy (Gallelli and Mangini, 1995)
DDE (ppb)	fat	28	100	294	395 ± 264.4	–	–	–	Italy (Gallelli and Mangini, 1995)

DDT total	fat – serum	785–4200	100–99	–	–	–	–	–	NHANES II (Murphy et al., 1983)
Dieldrin (ppb)	fat	28	88	22	26 ± 15.7	–	–	–	Italy (Gallelli and Mangini, 1995)
	fat – serum	785–4200	95–9	–	–	–	–	–	NHANES II (Murphy et al., 1983)
Endrin (ppb)	fat	28	72	34	36 ± 15.1	–	–	–	Italy (Gallelli and Mangini, 1995)
Aldrin (ppm)	fat	92	47	–	0.16 ± 0.31	–	–	1.8	Italy (Pavan et al., 1987)
Heptachlor (ppm)	fat	92	10	–	0.019 ± 0.08	–	–	0.6	Italy (Pavan et al., 1987)
	serum	4200	<1	–	–	–	–	–	NHANES II (Murphy et al., 1983)
Oxychlordane (ppb)	fat	28	52	29	34 ± 20.5	–	–	–	Italy (Gallelli and Mangini, 1995)
	fat – serum	785–4200	95–4	–	–	–	–	–	NHANES II (Murphy et al., 1983)
trans-Nonachlor	fat – serum	785–4200	97–6	–	–	–	–	–	NHANES II (Murphy et al., 1983)
Heptachloroepoxide (*ppb*)	fat	28	88	40	45 ± 23.2	–	–	–	Italy (Gallelli and Mangini, 1995)
	fat – serum	785–4200	96–4	–	–	–	–	–	NHANES II (Murphy et al., 1983)
Mirex	fat – serum	785–4200	<1–<1	–	–	–	–	–	NHANES II (Murphy et al., 1983)

9.2. Environmental reference values

The first major study for the definition of environmental reference values of pesticides was the National Human Exposure Assessment Survey (NHEXAS), conducted with the aim of providing the necessary data for estimating total exposure of the general population to a variety of chemical substance, such as pesticides, metals and volatile organic compounds (VOCs), found in the environment (Lebowitz et al., 1995; Robertson et al., 1999; Gordon et al., 1999).

A first pilot study was conducted in Arizona, a state with a wide range of climatic and geographic situations, ideal for studying exposure patterns. Arizona also offered a variety of scenarios of potential exposure, having cities, mines, farming areas and smaller communities. The aims of the study included:

– to document the presence, distribution and determinants of total exposure of the general population;
– to characterise the 90th percentile of total exposure for all contaminants;
– to monitor geographical and temporal variations in exposure through various environmental compartments;
– to evaluate the influence of different factors on total exposure;
– to analyse biomarkers of selected contaminants in blood and urine;
– to evaluate total exposure of disadvantaged minorities as subgroups of the general population.

Participants were selected in three stages:

1. a large group of families (about 1200) were approached and answered a descriptive questionnaire;
2. a subsample of participants (505 families) was identified and answered a basic questionnaire; samples of airborne and deposited household dust were collected for screening;
3. a further subsample of houses (179 families) were monitored extensively and samples of outdoor earth, house dust, drinking water, indoor and outdoor air, skin wipes, 24-hour diet, blood and urine were obtained. A questionnaire was answered and a daily journal was kept.

The pesticides of major interest were diazinone and chlorpyrifos used domestically to control insects and on lawns, in gardens and on wooden frames of houses. Exposure (skin contact, inhalation and ingestion) was therefore possible indoors and outdoors. Pesticides of secondary interest, not considered in this part of the study, were malathion and carbaryl which are largely ingested with food.

Sampling methods and analytical procedures are indicated in Tables 6a and 6b; the results are summarised in Tables 7a and 7b.

The data shows a higher %pos in indoor than outdoor samples, especially for chlorpyrifos. The authors observe that the frequency distribution of chlorpyrifos and diazinone in house dust have similar trends, suggesting similar types of use. Significant correlations were found between concentrations in indoor air and skin wipes and less significant ones between hand contamination and floor dust. This suggests that house dust contributes less to exposure of adults than air. Correlations

Table 6a. Sampling methods used in NHEXAS (Gordon et al., 1999).

Air sampling (flow 4 l/min, glass fiber filter and PUF*)	Outdoor: integrated 24-h sample over a 3-day period, in the backyard of the home, at least 3 m from the house, trees and walls. Indoor: integrated 12-h sample over a 3-day period, in the main living area of the home. Personal: integrated 8-h sample over a 1-day period
Floor dust sampling (a vacuum device specifically fabricated vas used)	Integrated collection of dust from a 4-m^2 area in the main living room and a 4-m^2 area in the primary respondent's bedroom (a 3-m^2 area was vacuumed in the center of the room and a 1-m^2 in accessible corners).
Window-sill wipe sampling (water-moistened gauze pads)	Sample composited by two wipes, one from the main living room and one from the bedroom.
Dermal wipe sampling (isopropanol-moistened gauze pads)	Collection from primary respondent's hands
Yard and foundation soil sampling (stainless steel trowel)	Composite sample collected from eight locations around the home (10 g of soil at each site from no more than 2.5-cm depth).

* Polyurethane foam.

Table 6b. Analytical procedures used in NHEXAS (Gordon et al., 1999).

Analytical procedure	• Addition of fenchlorphos (*surrogate recovery standard*); • extraction with Soxhlet technique or by sonication with acetone (solvent exchange in acetonitrile/esano on Extrelut for wipe tests); • purification by SPE (C18); • analysis by GC/ECD or GC/MS (internal standard trichloronate).
Quality Assurance/Quality Control	• Measure of SRS recovery; • analysis of duplicate samples; • analysis of field and laboratory blanks; • analysis of field and laboratory-spiked samples; • one matrix sample from each batch of pre-cleaned sampling media was analyzed before field use to ensure that material met acceptance criteria.

between concentrations of chlorpyrifos in house dust, and outdoor soil or foundation soil also had low significance. This confirms that indoor levels are largely derived from indoor use of pesticides and not pesticides from outside the house.

To conclude, it can be said that most exposure occurs inside the home: since chlorpyrifos has a moderate vapour pressure, after use (it is typically sprayed at floor level) it diffuses in the vapour phase and is absorbed by indoor airborne particulate. About 14% of the population monitored stated that they had not used chlorpyrifos in the house in the previous 6 months. This percentage was similar to the percentage of house dust samples with undetectable levels of the pesticide.

Table 7a. Results of NHEXAS – indoor sampling (Gordon et al., 1999).

Matrix		% Pos	No.	50%	75%	90%	100%
Floor dust (µg/g)	Chlorpyrifos	88	218	0.16	0.72	3.2	119
	Diazinon	53	218	–	–	–	66.2
Dermal wipe (µg/two hands)	Chlorpyrifos	36	149	0.003	0.029	0.207	544
	Diazinon	32	149	–	–	–	18.4
Indoor air (ng/m^3)	Chlorpyrifos	65	122	8	32	85	3280
	Diazinon	63	122	–	–	–	20500
Window-sill wipe (µg/m^2)	Chlorpyrifos	54	68	0.32	2.49	15.4	16100
	Diazinon	15	68	–	–	–	232
Personal air (ng/m^3)	Chlorpyrifos	17	6	–	–	–	175
	Diazinon	0	6	–	–	–	–

Table 7b. Results of NHEXAS – outdoor sampling (Gordon et al., 1999).

Matrix		% Pos	No.	100%
Yard soil (µg/g)	Chlorpyrifos	31	281	0.40
	Diazinon	50	281	4.9
Foundation soil (µg/g)	Chlorpyrifos	48	156	85
	Diazinon	57	156	7.0
Outdoor air (ng/m^3)	Chlorpyrifos	10	42	22.5
	Diazinon	9	42	131

10. ITALIAN STUDIES

10.1. Urinary alkylphosphates

Concentrations of alkylphosphates in urine reflect recent exposure to organophosphorus insecticides. All six alkylphosphates are metabolic products of various compounds. Table 8 shows the relation between metabolites excreted and the compounds from which they may be derived.

The biological origin of more than one pesticide prevents specific identification of the source of exposure. This means that information obtained from analysis of alkylphosphates in urine can only be used for screening unless the source of exposure is known, for example the work environment.

The first large study completed on this topic was NHANES II (Murphy et al., 1983). Conducted between 1976 and 1980 on 5976 urine samples of the American population, this study showed percentage positivities of the six alkylphosphates ranging from less than 1% to 12%. The analytical method used had a LOD of 20 µg/l. The authors do not report the concentrations measured in positive samples (Murphy et al., 1983).

The next two studies of interest were conducted in Italy in 1995 on the general adult (Aprea et al., 1996c) and child (Aprea et al., 2000) population. The analytical procedure used (Aprea et al., 1996a) had a LOD of 2–3 µg/l and enabled detection of at least one of the six analytes in all samples. Further studies were

Table 8. Alkylphosphate excreted in urine after exposure to various pesticides.

Pesticide	DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Azinphos-ethyl				x	x	x
Azinphos-methyl	x	x	x			
Chlorethoxyphos				x	x	
Chlorfenvinphos				x		
Chlormephos				x	x	x
Chlorpyrifos				x	x	
Chlorpyrifos-methyl	x	x				
Coumaphos				x	x	
Cyanophos	x	x				
Dichlorvos (DDVP)	x					
Diazinon				x	x	
Dicrotophos	x					
Dimethoate	x	x	x			
Disulfoton				x	x	x
Ethion				x	x	x
Fenitrothion	x	x				
Fensulfothion				x	x	
Fenthion	x	x				
Formothion	x	x	x			
Heptenophos	x					
Isazophos				x	x	
Isazophos-methyl	x	x				
Jodfenphos	x	x				
Malathion	x	x	x			
Methidathion	x	x	x			
Mevinphos	x					
Monocrotophos	x					
Naled	x					
Omethoate	x	x				
Oxydemeton-methyl	x	x				
Parathion				x	x	
Parathion-methyl	x	x				
Phorate				x	x	x
Phosalone				x	x	x
Phosmet	x	x	x			
Phosphamidon	x					
Phoxim				x	x	
Pirimiphos-ethyl				x	x	
Pirimiphos-methyl	x	x				
Prothoate				x	x	x
Pyrazophos				x	x	
Quinalphos				x	x	
Sulfotep				x	x	
Temephos	x	x				
Terbufos				x	x	x
Tetrachlorviphos	x					
Tolclofos-methyl	x	x				
Triazophos				x	x	
Trichlorfon	x					
Vamidothion	x	x				

conducted recently in Germany on 57 adults (Hardt and Angerer, 2000) and in the USA on 703 persons between 6 and 59 years of age (CDC, 2002). The American study is known as NHANES 1999–2001 and the results recently available on the web page of the National Center for Environmental Health, Centers for Disease Control and Prevention (CDC) of Atlanta (CDC, 2002) are for samples obtained in 1999.

The design and strategy of the more recent studies are shown in Tables 9a and 9b together with details of the analytical procedures. The results are shown in Table 10.

The %pos found in the Italian studies (Aprea et al., 1996c, Aprea et al., 2000) are much higher than those of NHANES II (Murphy et al., 1983), especially for dimethylated compounds. This difference cannot be attributed exclusively to the 10–20-fold lower LOD of the analytical method.

Table 11 shows the percentage of positive samples in the three studies for a LOD of 20 µg/l. A bias towards dimethylated metabolites is evident for the Italian studies and towards diethylated compounds for the American study.

These differences could depend on the different chemical structure of the phosphoric esters used in the two countries. Dimethylated phosphoric esters are generally regarded as less toxic than their diethylated equivalents, e.g. chlorpyrifos-methyl versus chlorpyrifos). The Italian data is, however, similar to the German results, though much lower median concentrations of DMP and DMTP were obtained in Italy. Similarly, the %pos found in the German study for DMDTP was nearly double that encountered in Italy: the authors state that most of the phosphoric esters used were insecticides, such as chlorpyrifos, dichlorvos, dimethoate, oxydemeton-methyl, parathion, parathion-methyl and pirimiphos-methyl; the only one of these that could give rise to DMDTP was dimethoate (Hardt and Angerer, 2000).

The Italian data (geometric mean) was about four-fold that of the last NHANES (CDC, 2002); the analytical procedure used is to be published soon, however the results suggest a LOD lower by a factor of 2–10 than LODs of the Italian and German studies.

Figure 1 shows the geometric means of the six alkylphosphates measured in adults and children in the Italian studies, expressed in nmol/g creat.

Excretion levels in children were significantly greater than in adults, presumably due to the greater exposure of children to residues in food (dietary exposure) and house dust (cutaneous and oral, non dietary exposure) (Aprea et al., 2000). The two Italian studies also report an evaluation of the influence of various confounding factors on urinary excretion of the six metabolites, demonstrating that of a whole series of variables (sex, age, sampling period, alcohol consumption, smoking, place of residence, food supply), only age (over or under 40 years) was significant against DMTP according to ANOVA, probably due to age-related differences in diet. For children, the multiple regression model, which included all the variables of the questionnaire (sex, house with garden/vegetable plot, cut flowers, ornamental plants, pets, school mensa serving organic food, spraying of pesticides in the previous month), showed statistically significant fitting of data for DMTP and DMDTP (Aprea et al., 2000).

Alkylphosphates are sensitive indicators of exposure to phosphoric esters: during

Table 9a. Design, strategy and analytical procedures used in the studies on urinary alkylphosphates.

	Italian adults (Aprea et al., 1996c)	Italian children (Aprea et al., 2000)	Germany (Hardt and Angerer, 2000)	NHANES 1999 (CDC, 2002)
No. samples	124 (93 M and 31 F)	195 (92 M and 103 F)	54 (48 M and 6 F)	703
Age of population (years)	16–59	6–7	22–57	6–59
Sample type	2nd morning urine (9–12 a.m.)		Urine spot samples	–
Sample conservation	Freezing (–18 °C)		Freezing (–18 °C)	–
Quality control/Quality assurance	Control cards on spiked samples		Spiked samples	–
Sampling period	1995		–	–
Sample containers	Polyethylene containers shielded from light; no preservatives or stabilizers added		Polypropylene containers	–
Sampling design	Sampling in a coastal and a mountain area of Tuscany. Location was not a confounding variable so the samples ware analyzed all together	Sampling in 1st and 2nd class of all the elementary schools in Siena centre; partecipation about 65%	Subjects living in East Germany, not occupationally exposed to organophosphorous compounds	–
Statistic analysis	Log trasformation of data to obtain normal distribution. ANOVA on factors investigated by questionnaire			–
Analytical procedure	Aprea et al. (1996a)		Hardt et al. (2000)	Not yet published

Table 9b. Analytical procedures for the determination of alkylphosphates in urine.

	Aprea et al. (1996a)	Hardt et al. (2000)	NHANES 1999*
Analytes	DMP, DMTP, DMDTP, DEP, DETP, DEDTP	DMP, DMTP, DMDTP, DEP, DETP, DEDTP	DMP, DMTP, DMDTP, DEP, DETP, DEDTP
Urine volume (ml)	2	5	–
Analyte isolation	Azeotropic distillation with acetonitrile	Extraction with ether/acetonitrile	–
Derivatization	PFBBr	PFBBr	–
Purification	SPE–CN	Addition of water and extraction with hexane	–
Apparatus	GC/FPD	GC/MS SIM	GC/MS/MS
IS	sulfotep	dibutyl phosphate	Stable isotopes
LOD (nmol/l)	9–18 nmol/l	1–5 µg/l	–
% Recovery	86–101	71–114	–
CV%	7.9–11.9[a] (1.9–4.8[b])	8.8–15.5[c] (7.9–17.0[d])	–

[a] whole analysis; [b] chromatographic analysis only; [c] within-series; [d] between-day.
PFBBr = pentafluorobenzylbromide
* Not yet published.

manual operations on ornamental plants treated with omethoate and/or fenitrothion (Aprea et al., 1994; Aprea et al., 1999b; Aprea et al., 2001), levels of excretion were found to be only slightly above data obtained in adults of the general population not occupationally exposed to these substances. Workers employed in industrial formulation of dimethoate (Aprea et al., 1998) excreted a quantity of metabolites 20 times greater than reference values, and cases of acute poisoning by voluntary ingestion had excretions 2–3 orders of magnitude greater (Aprea et al., 2001b).

10.2. Ethylenethiourea (ETU) in urine

Ethylenethiourea (ETU) is one of the human metabolic products of ethylenbis-dithiocarbamates (EBDCs) (WHO, 1988); it may be present as impurity in commercial EBDC-based formulations (Bontoyan and Looker, 1973; Bontoyan et al., 1972) or may form in the environment by biotic or abiotic degradation of these compounds (WHO, 1988). EBDCs in food may be transformed into ETU during industrial or domestic preparation of food (Watts et al., 1974; Newsome and Laver, 1973). Until September 2001, the International Agency for Research on Cancer (IARC) classified ETU in group 2B on the basis of evidence of carcinogenicity in animals but insufficient evidence of same in humans. IARC subsequently reclassified the molecule in class 3 (IARC, 1974; IARC, 1983).

A recent report of the Istituto Superiore di Sanità (Ministero della Sanità, 1998) showed that only seven of the 139 samples of fruit and vegetables analysed in 1997 contained EBDC residues in the range 1–20 mg/kg (four above legal limits). The vegetables most frequently contaminated were lettuce and endive. With regard to fruit, of the 209 samples analysed, seven contained residues in the interval 0.1–1.0

mg/kg, all within legal limits. The fruit most often contaminated included apples, apricots, peaches and pears. None of the three cereal samples analysed contained quantifiable residues.

These low values are in contrast with the widespread use of EBDC in Italian agriculture and are probably due to analytical problems in detecting trace quantities (µg/kg levels) of EBDCs and ETU. This may explain the presence of ETU in urine of the general population not professionally exposed to EDBCs.

The only studies completed on urinary assay of ETU in the general population were carried out in the framework of the activity of ISRV (Aprea et al., 1997a; Aprea et al., 1996b). The design and strategy of these studies are shown in Tables 12a and 12b together with details of the analytical procedure (Aprea et al., 1993). The results are summarised in Table 13 and Figure 2.

The analytical procedure (Aprea et al., 1993) had a LOD of 1 µg/l and detected the analyte in 24% of the 167 subjects not occupationally exposed to EBDC/ETU resident in four regions of central and northern Italy and 37% of samples from Rovescala, a wine producing area in the Pavia area treated yearly with EBDC by aerial spraying (Aprea et al., 1996b). In the two groups of subjects, variables with a significant influence on urinary concentrations of ETU were found to be wine consumption and tobacco smoking. Treatment of tobacco plants with EBDC may result in traces of ETU in cigarettes (8–27 ng/cig) (Autio, 1983) and analysis of ETU in wine has constantly revealed concentrations of ETU of 5–10 µg/l if a sufficiently sensitive method is used (Aprea et al., 1997a; Aprea et al., 1996b).

The influence of consumption of wine, fruit and vegetables on the presence of ETU in urine also emerged in a subsequent study with volunteers on a controlled diet (Aprea et al., 1997a). The aim of the study was to monitor urinary excretion of ETU in five male non smoker volunteers over a period of eight days. The volunteers were on a diet consisting of food and drinks with known ETU content. They took three meals per day together for eight days, eating the same quantity of the various foods offered. In the first two days their diet lacked wine, fruit and vegetables; in the next three days their diet also included these items. On days 6 and 7 they returned to the initial diet without wine, fruit and vegetables. On the last day their diet again contained wine, fruit and vegetables. Figure 2 shows the time course of excretion during biological monitoring. The pattern suggests that ETU is almost completely eliminated within 24 h of ingestion of ETU and EBDC residues. Indeed in urine excreted on day 6 (first day without wine, fruit and vegetables after three days of their consumption) concentrations of ETU were close to the LOD in all samples. The pattern of excretion also suggests that ETU and EBDC intake was extremely limited on days when these items were not eaten.

The results of the ISRV studies (Aprea et al., 1997a; Aprea et al., 1996b) were similar to those of two control groups used in studies of workers occupationally exposed to EBDC, living near areas treated with fungicides: %pos were 91% and 30% in spring–summer (spraying time) and autumn–winter, with concentration intervals of 2.0–10.1 µg/l and 2.2–4.1 µg/l, respectively (Sciarra et al., 1994).

Urinary ETU is a sensitive indicator of exposure to EBDC: excretion of ETU by workers engaged in industrial formulation of mancozeb has been found to be

Table 10. Results of studies on urinary alkylphosphates (µg/l).

Analyte	% Pos	No.	Mean±SD	GM	10%	25%	50%	75%	90%	95%	100%	Study (Reference)
DMP	87	124	12.03 ± 11.58*	7.65*	<LOD	4.13	9.07	15.68	27.00	35.04	70.71	Italian adults (Aprea et al., 1996c)
	96	195	18.17 ± 27.87*	10.22*	3.03	5.61	9.92	20.03	36.42	48.27	231.77	Italian children (Aprea et al., 2000)
	12	5976	–	–	–	–	–	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	1.84	<LOD[1]	0.80	1.67	3.79	7.43	–	–	USA NHANES (CDC, 2002)
	96	54	–	–	–	–	30	–	–	105	322	Germany (Hardt and Angerer, 2000)
DMTP	99	124	20.91 ± 21.66*	13.11*	3.22	6.72	13.03	30.20	45.73	63.08	129.84	Italian adults (Aprea et al., 1996c)
	94	195	18.92 ± 23.96*	10.29*	2.50	5.04	10.36	20.97	49.96	67.34	164.69	Italian children (Aprea et al., 2000)
	6	5976	–	–	–	–	–	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	2.61	<LOD[2]	0.72	3.80	9.00	22.9	–	–	USA NHANES (CDC, 2002)
	100	54	–	–	–	–	22	–	–	174	324	Germany (Hardt and Angerer, 2000)
DMDTP	48	124	3.45 ± 4.99*	1.86*	<LOD	<LOD	<LOD	4.63	7.36	11.60	30.31	Italian adults (Aprea et al., 1996c)
	34	195	3.51 ± 7.61*	1.55*	<LOD	<LOD	<LOD	3.58	9.43	13.84	90.61	Italian children (Aprea et al., 2000)
	<1	5976	–	–	–	–	–	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	0.51	<LOD[3]	<LOD[3]	0.60	2.05	5.43	–	–	USA NHANES (CDC, 2002)
	89	54	–	–	–	–	1	–	–	8	51	Germany (Hardt and Angerer, 2000)

												Reference
DEP	82	124	8.57 ± 12.19*	4.80*	<LOD	1.58	5.01	9.37	17.60	27.36	93.08	Italian adults (Aprea et al., 1996c)
	75	195	5.71 ± 6.71*	3.55*	<LOD	<LOD	3.84	7.07	12.49	18.58	47.69	Italian children (Aprea et al., 2000)
	7	5976	–	–	–	–	–	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	2.55	0.78	1.09	1.85	4.87	10.6	–	–	USA NHANES (CDC, 2002)
	94	54	–	–	–	–	4	–	–	21	46	Germany (Hardt and Angerer, 2000)
DETP	73	124	5.39 ± 6.18*	3.18*	<LOD	<LOD	3.60	6.75	17.60	27.36	37.18	Italian adults (Aprea et al., 1996c)
	48	195	3.87 ± 5.75*	1.89*	<LOD	<LOD	<LOD	4.16	9.64	16.46	30.98	Italian children (Aprea et al., 2000)
	6	5976	–	–	–	–	–	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	0.81	0.51	0.58	0.70	0.98	1.52	–	–	USA NHANES (CDC, 2002)
	46	54	–	–	–	–	<LOD	–	–	15	55	Germany (Hardt and Angerer, 2000)
DEDTP	7	124	1.03 ± 0.98*	0.91*	<LOD	<LOD	<LOD	<LOD	<LOD	1.73	9.60	Italian adults (Aprea et al., 1996c)
	12	195	1.45 ± 1.97*	1.00*	<LOD	<LOD	–	<LOD	3.11	4.40	20.84	Italian children (Aprea et al., 2000)
	<1	5976	–	–	<LOD	0.09	0.30	–	–	–	–	USA NHANES II (Murphy et al., 1983)
	–	703	–	0.19	0.08	–	0.14	–	0.43	–	–	USA NHANES (CDC, 2002)
	2	54	–	–	–	–	<LOD	–	–	<LOD	19	Germany (Hardt and Angerer, 2000)

* Values obtained by substituting half LOD for undetectable concentrations.   [1] LOD = 0.51 µg/l;   [2] LOD = 0.18 µg/l;   [3] LOD = 0.08 µg/l.

Table 11. Percentage of positive analyses for urinary alkylphosphates from American and Italian studies considering the same LOD of 20 μg/l.

	DMP %pos	DMTP %pos	DMDTP %pos	DEP %pos	DETP %pos	DEDTP %pos
NHANES II (Murphy et al., 1983)	12	6	<1	7	6	<1
Italian adults (Aprea et al., 1996c)	15	32	3	6	4	0
Italian children (Aprea et al., 2000)	26	28	2	0.5	2	0.5
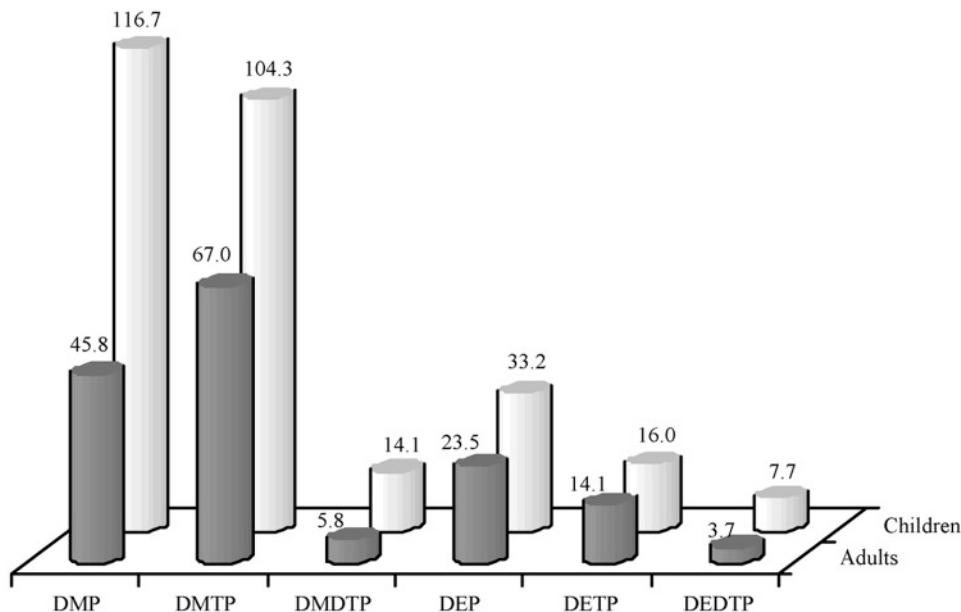
Figure 1. Urinary excretion of alkylphosphates (nmol/g creat) in the Italian studies (Aprea et al. 1996c, Aprea et al., 2000). Values reported as geometric mean.

5–30 times greater than in the general population (Aprea et al., 1998), and in agricultural workers engaged in spraying, this figure rises by a factor of 10–100 (Kurttio et al., 1990; Kurttio and Savolainen, 1990).

10.3. 3,5,6-trichloro-2-pyridinol (TCP) in urine

TCP, the specific metabolite of chlorpyrifos (CP) and chlorpyrifos-methyl (CPM) is excreted in urine as glucuronate (Nolan and al., 1984; Richardson, 1995; Sultatos et al., 1984) and has been assayed in various occupational situations (Aprea et al., 1997c, Fenske and Elkner, 1990) and in the general population (Aprea et al., 1999a; Hill et al., 1995b; Kutz et al., 1992; Hill et al., 1989).

A recent report of the Istituto Superiore di Sanità (Ministero della Sanità, 1998) shows that of 2448 samples of vegetables analysed for CP residues in 1997, 22

Table 12a. Design, strategy and analytical procedures used in the Italian studies on urinary ETU.

	Study no. 1 (Aprea et al., 1996b)	Rural population (Rovescala)	Study no. 2 (Aprea et al., 1997a)
Population residence	Urban population (Pavia, Torino, Trento e Verona)	Rural population (Rovescala)	Urban population (Pavia)
No. samples	167 (120 M and 47 F)	97 (50 M and 47 F)	Five male volunteer non-smokers
Age of population (years)	17–61	22–65	26–34
Sample type	2nd morning urine (9–12 a.m.)	Spot urine samples	24 h urine samples in a single container or in three separate container, one for each 8-h period
Sample conservation	Freezing (the sample was stable at least 350 days)		
Quality control/ Quality assurance	Interlaboratory controls on spiked samples (Youden test); analysis done in five laboratories using the same analytical procedure		
Sampling period	1994–1995	1993	1996
Sample containers	Polyethylene containers shielded from light; no preservatives or stabilizers added		
Sampling design	Subjects of the general population coming for check-ups to the medical centres (special questionnaire)	Subjects living in a hillside wine-producing town, where mancozeb spaying was being performed by helicopter	The volunteers took meals together during the 8 days of the study, and ate the same quantities of each food. Days 1, 2, 6, 7 no wine, vegetables and fruit. Days 3, 4, 5, 8 wine, vegetables and fruit were included in the menu.
Statistic analysis	Performed by the χ^2 test, dividing the data into two groups according to whether or not they contained detectable ETU levels (to evaluate the influence of age, residence, sex, smoking and wine consumption). Performed by the logistic regression (Wald test) to evaluate the influence of all variables together.		Mean urinary excretion of ETU in the five volunteers during 8-day monitoring study.
Analytical procedure	Aprea et al. (1993)		

Table 12b. Analytical procedures for the determination of ETU in urine (Aprea et al., 1993).

Sample volume (ml)	18
Sample preparation	Addition of NH_4Cl and KF
Analyte isolation	Extraction on Extrelut with dichloromethane
Purification	SPE (silica)
Apparatus	HPLC/DAD (232 nm)
IS	–
LOD ($\mu g/l$)	1.0
Recovery%	91.1 ± 8.9 (6.9 $\mu g/l$)
CV%	9.8% (6.9 $\mu g/l$)

Table 13. Urinary concentration of ETU ($\mu g/l$) in the general population (Aprea et al., 1996b).

Population	%pos	Mean±SD	GM	50%	75%	90%	95%	100%
Urban	24	1.3 ± 1.8*	0.8*	<LOD	<LOD	3.5	5.3	10.0
Rural	37	4.1 ± 9.5*	1.3*	<LOD	4.5	8.1	16.5	63.2

* Values obtained by substituting half LOD for undetectable concentrations.
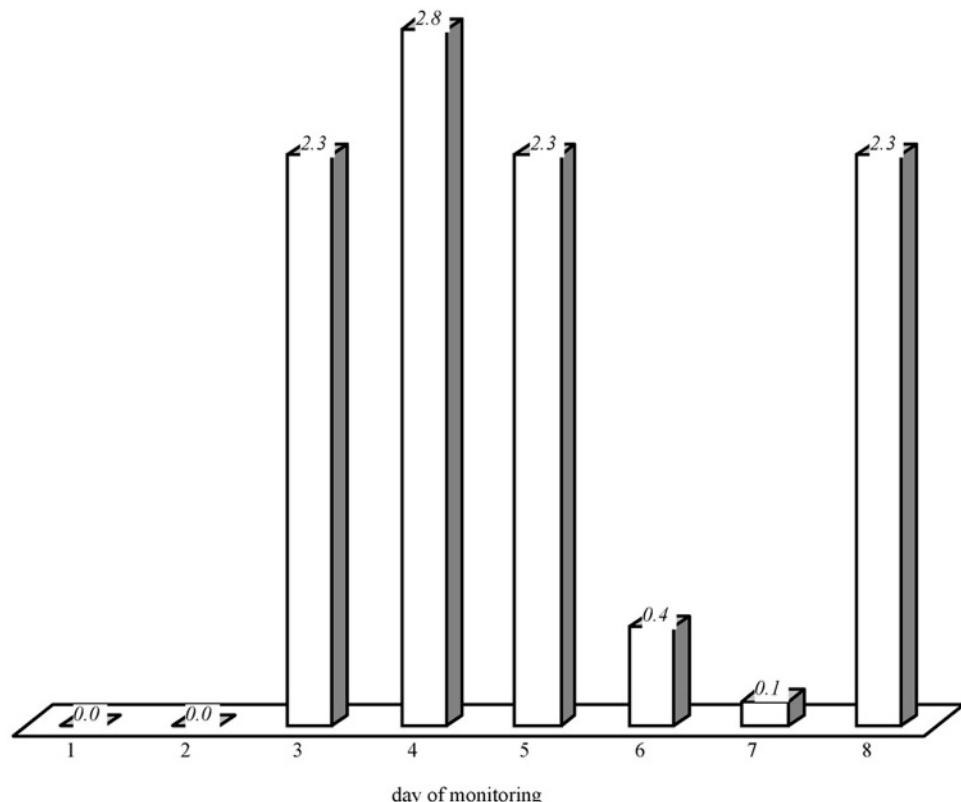

Figure 2. Mean urinary excretion of ETU ($\mu g/24$ h) in five volunteers on a controlled diet (Aprea et al. 1997a).

contained residues but were within legal limits and of the 2145 samples analysed for CPM, 11 contained residues and one sample of celery was above legal limits. Out of 3013 samples of fruit analysed for CP, 5.2% contained residues (within legal limits); 86 of the 2657 fruit samples analysed for CPM contained residues and only one mandarin sample was above legal limits. Of the 172 cereal samples analysed for CP only two contained quantifiable residues; of the 152 analysed for CPM, six contained residues within legal limits.

Considering the widespread use of CP and CPM and the non negligible presence of residues in food, ISRV conducted a first study to determine TCP in urine of the general population (Aprea et al., 1999a) This study also carried out interlaboratory controls of the analytical procedure used. TCP concentrations were determined in 42 urine samples of the general population of central-northern Italy. The study was subsequently extended (data not published) to examine the influence of certain variables (town and season) on urinary excretion of TCP. The design and strategy of the two studies are shown in Tables 14a and 14b, together with details of the analytical procedure. The results are summarised in Table 15. The data collected in 1997 demonstrated a significant influence of wine consumption and a prevalently vegetarian diet on urinary excretion of TCP (Aprea et al., 1999a). The data collected in 1998 showed lower excretion than 1997 and the population of the city of Turin was found to be less exposed than residents of Novafeltria. Excretion was not found to be influenced by the season of sampling (summer/winter). The variable 'wine consumption' was confirmed to be statistically significant, also in the complete series of 109 urine samples obtained in summer and could partly explain the differences obtained between Turin (70% non drinkers) and Novafeltria (53% non drinkers) but not between the data of 1997 (65% drinkers) and 1998 (60% drinkers). Differences between the two years could be due to a real reduction in CP and CPM residues in food.

The results of the ISRV study can be compared with those obtained in a control group used in the course of a study of workers occupationally exposed to CPM, and in whom a %pos of 78%, geometric mean concentration of 2.6 µg/l and maximum of 12.1 µg/l were found (Aprea et al., 1997b). The percentage of positive samples found in the ISRV studies was much higher than in NHANES II: this difference may be partly due to the different LODs of the analytical methods used, however 18% of the ISRV samples were above 5 µg/l as against 5.8% in the American study (Kutz et al., 1992). The values encountered in the ISRV studies were only slightly below those of NHANES III: this difference may be due to the widespread use of CP as domestic insecticide in the USA (Hill et al.1995b).

Urinary TCP is a sensitive and specific indicator of exposure to CP and CPM. Excretion of TCP by workers exposed to CPM in vineyards was 3 (green pruning) and 20 (mixing and spraying) times greater than that of the general population (Aprea et al., 1997b). Excretion levels of the same order of magnitude were recorded for workers engaged in treatment of house structural frameworks with CP (Fenske and Elkner, 1990).

Table 14a. Analytical procedure for the determination of 3,5,6-trichloro-2-pyridinol in urine (Aprea et al., 1999a).

Sample volume	10 ml	
Hydrolysis	Hot HCl	
Analyte isolation	Extraction with toluene	
Derivatization	BSA	
(1 h room temperature)		
Purification	–	–
Apparatus	GC/ECD	GC/MS SIM (m/z 254 and 256 for TCP, m/z 181 and 183 for IS)
IS	γ-hexachlorocyclohexane	γ-hexachlorocyclohexane and 2,4,6-TCPh
LOD μg/l	1.2	1.5
% recovery	–	–
CV%	8.2	–

BSA = N,O-bis(trimethylsilyl)acetamide; 2,4,6-TCPh = 2,4,6-trichlorophenol.

11. OTHER STUDIES

Various other assays of metabolites of pesticides in biological samples of the general population have been done in the course of studies of occupationally exposed subjects. In these cases, the subjects monitored figure as control groups and the results obtained are not accompanied by a study of the variables to evaluate the influence of diet, life-style or luxury consumer items on urinary excretion of blood levels.

By way of example, Table 16 summarises results obtained for urinary TTCA and free and total carbon sulphide in subjects not occupationally exposed to pesticides.

The two groups monitored acted as control groups used in an assessment of occupational exposure to alkylenbisdithiocarbamates (Weiss et al., 1999) and dithiocarbamates (Brugnone et al., 1993): in both cases, exposed subjects showed concentrations of analyte 2–3 times greater than subjects not occupationally exposed.

12. CONCLUSIONS

To conclude the present paper, it is worth stating that evaluation of the presence of xenobiotics in biological fluids of the general population is an excellent indicator of ubiquitous environmental contaminants and is more sensitive than evaluation of contaminants in environmental matrices (such as air, water, food, drinks). Indeed, if results above detection limits are not obtained in environmental matrices or food, there is the risk of drawing the erroneous conclusion that these substances are not widespread in the environment and that they are therefore not dangerous for humans or life on our planet in general. The fact that detection limits are not reached in food or the environment does not mean that xenobiotics are absent. Xenobiotics may be found in increasing concentrations in living organisms, the higher they are in the food chain. Since organisms do not have a direct relation

Table 14b. Design, strategy and analytical procedures used in the ISRV 's studies on urinary TCP.

	ISRV Study no. 1 (Aprea et al., 1999a)	ISRV Study no. 2*
Population residence	Urban (Pavia, Siena and Trento)	Urban (Novafeltria and Torino)
No. samples	42 (21 M and 21 F)	107
Age of population (years)	22–52	21–57
Sample type	2nd morning urine (9–12 a.m.)	
Sample conservation	Freezing (the sample was stable at least 40 days)	
Quality control/Quality assurance	Interlaboratory controls on spiked samples. Analysis done in two laboratories using different instrumental techniques	In the analytical serie
Sampling period	1997	1998
Sample containers	Polyethylene containers shielded from light; no preservatives or stabilizers added	
Sampling design	Subjects of the general population (special questionnaire). Exclusion criteria for participants: smoke <5 cigarettes/day; wine <250 ml/day; no medicines in the last month; no use of agricultural chemicals in the last year	Subjects of the general population (special questionnaire). 40 subjects from Novafeltria have been sampled in two sono stati campionati in due periodi dell'anno (winter and summer) per valutare l'influenza della stagione di prelievo
Statistic analysis	ANOVA on factors investigated by questionnaire	Statistical comparison on the base of variables obtained with the questionnaire
Analytical procedure	Aprea et al. (1999a)	

* Not yet published.

Table 15. Results of Italian studies on urinary TCP (μg/l).

Population	% Pos	No.	Mean ± SD	GM	10%	25%	50%	75%	90%	95%	100%	Study (Reference)
Pavia, Siena, Trento (1997 summer)	88	42	4.1 ± 3.1*	3.0*	<LOD	1.4	3.5	5.4	7.2	13.2	14.7	(Aprea et al., 1999a)
Novafeltria (1998 summer)	78	40	2.77 ± 2.1*	2.1*	<LOD	1.4	2.6	3.5	5.3	6.4	11.1	(unpublished data)
Novafeltria (1998 winter)	72	40	3.1 ± 3.2*	2.0*	<LOD	<LOD	2.3	4.1	5.8	11.7	14.2	(unpublished data)
Torino (1998 summer)	48	27	1.5 ± 1.8*	1.1*	<LOD	<LOD	<LOD	1.7	2.7	3.9	9.8	(unpublished data)
Pavia, Siena, Trento, Novafeltria, Torino (1997–1998 summer)	74	109	3.0±2.8*	2.0*	<LOD	<LOD	2.2	3.6	6.0	7.5	14.7	(unpublished data)

* Values obtained by substituting half LOD for undetectable concentrations.

Table 16. Urinary excretion of TTCA (μg/l) and blood concentration of carbon disulphide (ng/l), in two control groups monitored during the evaluation of occupational exposure to alkylenebisdithiocarbamates (Weiss et al., 1999) and dithiocarbamates (Brugnone et al., 1993) respectively.

Analyte	No.	%pos	Mean ± SD	GM	50%	95%	100%
TTCA in urine	50	96	35 ± 44	–	16	123	170
CS2 free in blood	112	100	663 ± 71	414	453	–	5339
CS2 total in blood	112	100	3178 ± 282	2254	2066	–	13575

with any single matrix but with various matrices, they function as 'concentrator-accumulators'. From this viewpoint, since humans are at the centre of 'industrial civilisation' and at the top of the food chain, they can be regarded as one of the best available indicators of widespread contamination.

These considerations cast doubt on the effective possibility of defining 'reference exposure conditions' of the general population to pesticides widely used today.

APPENDIX 1

QUESTIONNARIE FOR THE DEFINITION OF REFERENCE VALUES IN ADULT SUBJECTS (all answers will be treated as confidential)

Questionnaire no.	DATE

GENERAL DATA

Surname	Name

Sex	Date of birth

Place of birth	Address

Employer	Telephone number

PLACE OF RESIDENCE

Classification area of residence

Large town/city (> 10,000 persons)	Town (< 10000 persons)
Urban outskirts (small town, village)	Rural
Plain	Hills
Mountains	Lakeside
Seaside	

Nearby industry or agriculture	
Type	Distance from home (km)

OCCUPATION

Sector

Agriculture	Industry
Trade	Commerce
Government	Work at home
Housewife	

Qualification

Blue collar worker	Self-employed
White collar worker	Other (give details)
Pensioner	

Type of firm and activitiy	

Classification of area of work place

Large town/city (> 10,000 persons)	Town (< 10000 persons)
Urban outskirts (small town, village)	Rural
Plain	Hills
Mountains	Lakeside
Seaside	

Nearby industry or agriculture	
Type	Distance from workplace (km)

ESPOSURE TO PESTICIDES

Have you used pesticides in the last 3 months?	
Name of products and quantity?	
Reason for pesticide use?	
Use in course of part time or full time employment?	
Use outside work?	
Any hobby that may determine exposure to pesticides?	

DETAILED DATA

Do you have a garden or vegetable plot?		yes	no
If so, do you cultivate it yourself?			
yes	no	what jobs?	

Are there ornamental plants in the house?		
yes	no	
If so, do you treat them with chemical products?		
yes	no	which?

Do you often buy or handle cut flowers?	
yes	no

Have you done any of the things mentioned in the last three points in the last week?	
yes	no

Do you keep pets in the house?		
yes	no	which?
If so, have they been treated with pesticides (products against fleas/ticks in the case of dogs and cats) in the last month?		
yes	no	name of products?
Have you used insecticides or other products for woodworm or moth (spray, dust, liquid, bar) in the house in the last month?		
yes	no	which?

EATING HABITS AND LUXURY CONSUMPTION

Main type of diet	
mixed	Mainly vegetarian
Largely fish-based	

Eating habits

	Type	Quantity/day (hg)	Source of supply
Fruit*			
Vegetables*			
Meat**			

* specify whether home grown or bought at greengrocer's, supermarket, neighbourhood or other;

** specify whether home produced or bought from butcher, supermarket or elsewere.

DRINKING WATER

Indicate whether you mainly drink town water or mineral water and which brands.		
Source		
Town water	Privale well	Tank or source	Other
Indicate any analytical data on traces of pesticides in town water		

SMOKING OF TOBACCO

yes	no
I have smoked since (year)	
I stopped smoking in (year)	
Number of cigarettes per day	
< 5	5 - 10
10 – 20	> 20
Number of cigars per day	
Pipe smoker	yes no
Brand of cigarettes/cigars/tobacco	

WINE AND ALCOHOL CONSUMPTION

Wine	yes no
< 250 ml/day	< 500 ml/day
500-1000 ml/day	> 1000 ml/day
Source of wine	
Own or local production	
Bought in shop or supermarket	
Spirits	yes no
(indicate type and quantity/day)	
Other drinks	
coffee	yes how much no
tea	yes how much no
other (specify)	yes how much no

PERSONAL DATA FROM MEDICAL EXAMINATION AND BLOOD CHEMISTRY

Body weight (kg)	Height (cm)

Observations of examining doctor?	

APPENDIX 2

QUESTIONNARIE FOR THE DEFINITION OF REFERENCE VALUES IN
CHILDREN
(all answers will be treated as confidential)

Questionnaire no.	DATE

GENERAL DATA

Surname	Name

Sex	Date of birth

Body weight (Kg)	Height (cm)

Address	Telephone number

School	Class

Father occupation	Mother occupation

Occupation of other family members (specify)

Illness and hospitalization of child (specify)

Are there smokers in the house?
If so, how much?

Mean daily consumption of cigarettes in smokers?								
1st Subject (cigarettes/day)	< 5		5-10		11-20		>20	
2nd Subject (cigarettes/day)	< 5		5-10		11-20		>20	
3rd Subject (cigarettes/day)	< 5		5-10		11-20		>20	

DETAILED DATA

Do you have a garden or vegetable plot?	yes	no
If so, do you perform treatments of plants with chemical products (pesticides, fertilizers, other)?		
yes	no	which?

Are there ornamental plants in the house?		
yes	no	
If so, do you treat them with chemical products?		
yes	no	which?

Are there often cut flowers in the house?	
yes	no

Have been performed in the house any of the things mentioned in the last three points in the last week?	
yes	no

Do you keep pets in the house?		
yes	no	which?
If so, have they been treated with pesticides (products against fleas/ticks in the case of dogs and cats) in the last month?		
yes	no	name of products?
Have you used insecticides or other products for woodworm or moth (spray, dust, liquid, bar) in the house in the last month?		
yes	no	which?

DRINKING WATER

Indicate whether you mainly drink town water or mineral water and which brands.			
Source			
Town water	Privale well	Tank or source	Other
Indicate any analytical data on traces of pesticides in town water.			

WINE CONSUMPTION REFERED TO CHILD

yes	no	ml/day

Source of wine
Own or local production
Bought in shop or supermarket

OTHER DRINKS CONSUMPTION REFERED TO CHILD

Coffee
yes	no	ml/day

Thé
yes	no	ml/day

Other (specify)
yes	no	ml/day

FOOD AND DRINK INGESTED THE DAY BEFORE URINE COLLECTION

	Type	Quantity per day (g or ml)	Source of supply
Fresh fruit*			
Raw vegetables*			
Meat**			
Milk**			
Cheese**			
Bread			
Pasta			
Rice			
Cooked vegetables or legumes**			
Fish			
Eggs**			
Cold cuts			
Tinned foods			
Cooked fruit**			
Fruit juice			
Drinks			
Sweets**			
Dressing and condiment***			
Coca Cola			

*specify whether home grown or bought at greengrocer's, supermarket, neighbourhood or other;
** specify whether home produced or bought from butcher, supermarket or elsewere;
***olive oil, other oils, margarine, butter, spices, other.

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